

University of Alberta

**The Regulation of LFA-1-dependent Adhesion by
NK Activating Receptors**

by

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Abstract

NK cells play an integral part in host innate responses to tumors and viruses. Mouse Ly-49 receptors regulate NK cell effector responses through either the positive actions of activating receptors, or from negative signals from inhibitory receptors. Both activating and inhibitory Ly-49 family members recognize specific alleles of MHC I, or MHC I-like proteins. MHC I allele specificity is governed, in part, by residues within the $\beta 4$ - $\beta 5$ loop in the Ly-49 ectodomain, although the role of the $\beta 4$ - $\beta 5$ loop has not been previously established for the inhibitory Ly-49G. The CK-1 antibody recognizes amino acid polymorphisms within the ectodomains of C57BL/6 and BALB/c *Ly-49G* allele products. I identified the epitopes recognized by the Ly-49G specific antibodies CK-1 and Cwy-3. CK-1 and Cwy-3 epitopes mapped within the $\beta 4$ - $\beta 5$ loop and the $\beta 1$ strand, respectively, and were non-overlapping. Although both antibodies specifically recognized the Ly-49G^{B6} ectodomain, Cwy-3 was unable to block its interaction with MHC I, while CK-1 significantly inhibited it, supporting the idea that the $\beta 4$ - $\beta 5$ loop plays a key role in MHC I recognition. Ligand recognition by activating Ly-49 receptors on NK cells results in target cell lysis and cytokine production, yet how they mediate such activities is not well understood. I demonstrated that these receptors trigger LFA-1 dependent tight conjugation between NK cells and target cells. I also showed that activating Ly-49 receptor engagement leads to a rapid DAP12-dependent transient up-regulation of NK cell LFA-1 affinity for ICAM-1 that is also dependent on the activities of Syk and Src kinases. In addition, I demonstrated that these receptors can substantially increase

LFA-1-dependent tight adhesion to cell-sized beads immobilized with low ICAM-1 densities. Furthermore, I demonstrated that NKG2D/DAP10, NKRP-1C/FcR- γ and CD16/FcR- γ can also regulate LFA-1-dependent adhesion to ICAM-1, although these receptors were not as potent as activating Ly-49 receptors. I propose that enhanced binding LFA-1 to ICAM-1 mediated by NK activating receptors may be a mechanism employed by NK cells to reduce the threshold required for cell activation and to induce effector responses.

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List of abbreviations

Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
ADAP	adhesion and degranulation promoting adapter protein
APC	allophycocyanin
BCR	B cell receptor
B6	C57BL/6
β 2m	β 2-microglobulin
Ca^{++}	divalent calcium ions
CD3 ζ	CD3 zeta signaling adapter chain
^{51}Cr	$\text{Na}^{51}\text{CrO}_4$
CRD	carbohydrate recognition domain
CTL	cytotoxic T lymphocyte
DAP12	DNA-X protein of 12 kDa
DAP10	DNA-X protein of 10 kDa
DNAM-1	DNA-X activating molecule-1
E/T ratio	effector to target ratio
FCS	heat inactivated fetal calf serum
FSC	forward scatter
FcR- γ	Fc gamma receptor signaling adapter
Grz	granzyme
GPI	glycerophosphatidyl inositol
g α m	goat anti-mouse IgG
g α r	goat anti-rat IgG
HC	heavy chain
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HR	hybrid resistance
HSC	hematopoietic stem cell progenitor
Ig	immunoglobulin
IC	immune complex
ICAM-1	intercellular adhesion molecule-1
IFN γ	interferon
IL	interleukin
IL-2R β	interleukin 2 receptor beta chain
IL-15	interleukin 15
IL-15R α	interleukin 15 receptor alpha chain

iNK	immature natural killer cell
i.p.	intraperitoneal
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine based inhibitory motifs
IS	immunological synapse
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
KIR	killer cell immunoglobulin-like receptor
LAT	linker of activation in T cells
LFA-1	leukocyte function adhesion molecule 1
LRC	leukocyte receptor complex
mAb	monoclonal antibody
MCMV	mouse cytomegalovirus
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mNK	mature NK cell
NK	natural killer cell
NKG2D-L	long isoform of NKG2D
NKG2D-S	short isoform of NKG2D
NOD	non-obese diabetic
NTAL	non-T cell activation linker
PDB	protein database
pDC	plasmacytoid dendritic cell
PI3K	phosphoinositide-3- kinase
PLC- γ	phospholipases C gamma
poly I:C	poly inosinic acid:cytidylic acid
Prp	proline-rich protein
RapL tissue	regulator cell adhesion and cell polarization enriched in lymphoid
SCID	severe combined immunodeficiency
SFK	Src-family kinase
SHP-1	SH2 domain containing phosphatase-1
SHP-2	SH2 domain containing phosphatase-2
SHIP	SH2 domain inositol phosphatase
sICAM-1-Fc	dimeric soluble ICAM-1-Fc fusion protein
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SSC	side scatter
Syk	splenocyte tyrosine kinase

TAP	transporter of antigen processing
TcR	T cell receptor
TNF- α	tumor necrosis factor alpha
TRAIL	tumor necrosis factor related apoptosis inducing ligand
VLA-2	very late antigen-2
ZAP-70	zeta chain associated protein of 70 kDa

Chapter 1 - Introduction

A. Defining a Natural Killer Cell

The ongoing definition of natural killer (NK) cells has transformed over time from a simple activity, to a diversity of functions coupled with a still evolving repertoire of expressed cell surface markers. NK cells were first functionally identified as cells derived from mouse spleens that can efficiently lyse Moloney leukemia cells without prior sensitization (1, 2). From this observation, they were deemed as "naturally occurring killer cells" or natural killer cells. Although NK cells, like T cells, originate in the bone marrow; they mature in mostly a thymus-independent fashion (3, 4). In fact, unlike T or B cells, NK cells lack the expression of clonally distinct receptors that recognize a particular antigen. Moreover, mice that are deficient in the RAG-1 and RAG-2 recombinase genes do not develop mature B or T cells, as these enzymes are required for the rearrangement of antigen specific clonotypic receptors, whereas the NK cell compartment remains intact (5), (6).

In humans, NK cells are identified as being CD3 negative lymphocytes that express the molecule CD56 on their surface (7). CD56 is an N-linked glycoprotein that is expressed pre-dominantly on the surface of NK cells and some T lymphocytes (8). CD56 is a member of the human neural-cell adhesion molecule (N-CAM) family, and CD56 does not appear to have a clear function on human NK cells (8, 9). Human NK cells can be classified into two major populations based upon the cell-surface density of CD56 (7). Most NK cells in the blood (approximately 90%) have a lower density of CD56 (CD56^{dim}) and are highly cytotoxic, whereas higher expressing cells, or CD56^{bright} cells are less cytotoxic, but produce large amounts of cytokines following activation (10). In addition, CD56^{bright} cells can differentiate into CD56^{dim} cells. In contrast to human NK cells, CD56 is not a good pan-NK surface marker in mice. Instead, the NK1.1 antigen, recognized by the PK136 antibody antigen now the NKR1-1C receptor, is used to define murine NK cells in mouse strains such as C57BL/6 (B6). However, some mouse strains, such as BALB/c, do not express NKR1-1C on the surface of their NK cells. Consequently, the marker DX5, or CD49b, is

employed as a pan-NK marker as it is more widely expressed on mature mouse NK cells and some T cells. DX5 corresponds to the integrin CD49b/VLA-2 (very late antigen-2, $\alpha 2$ -integrin) which is expressed on many cell types, and is known to bind to collagen or laminin, although binding of NK cells to collagen is not blocked by DX5 mAbs (11). In addition, DX5 does not appear to regulate NK cell activation in mouse NK cells (11). Moreover, unlike CD56, it is not clear whether the levels of cell surface DX5 are indicative of functional differences within NK cell subsets *in vivo*.

NK cells have been shown to play a critical role in controlling viral infections in both mice and humans. For example, an adolescent patient with a germline NK cell deficiency developed a primary life-threatening human cytomegalovirus (HCMV) infection despite normal T and B cell functions (12). Similarly, NK cells play an integral role in regulating mouse cytomegalovirus (MCMV) infections. For example, the beige mouse, which lacks a functional inducible cytolytic NK response, is highly susceptible to MCMV infections (13). Importantly, *in vivo* depletion of NK cells using antibodies against the NK marker NK1.1 in B6 mice results in a marked increase in infection with MCMV (14). From the observation that NK cells specifically destroy tumor cells without prior sensitization, and the important role they play in regulating Herpes viral infections, many subsequent studies have been conducted to shed light on cell recognition and effector functions of NK cells.

B. NK Effector Functions

NK cells provide immunity to viral infections and help minimize the dissemination of tumors by their ability to destroy 'altered' cells following their interaction with activated dendritic cells which 'prime' NK responses through an IL-15 dependent mechanism (1, 2, 15, 16). They eliminate cells through a variety of mechanisms which include the perforin/granzyme pathway, the FasL/Fas or other death receptor interactions, and the release of a plethora of cytokines. Collectively, these mechanisms place NK cells in the forefront for controlling infections and destroying tumors.

NK cells are large granular lymphocytes that are not restricted by major histocompatibility complex (MHC) molecules for recognition (17). NK cells and cytotoxic T cells (CTL) have electron-dense cytotoxic granules within their cytoplasm. These lytic granules contain the protein perforin and a family of proteases known as granzymes, which upon exocytosis induce the destruction of the target cells. The degranulation, or exocytosis, of these granules is a contact-dependent mechanism (18, 19). Following target cell recognition, tight adhesion between the NK cell and the target cells leads to the aggregation of a number of receptors, and the formation of an organized structure within the membrane known as the immunological synapse (IS) (20). At the synapse, granules are delivered in a polarized fashion toward the target cell. Perforin promotes the delivery of granzymes into the target cell cytosol which ultimately induce the death of that cell via apoptosis (Fig. 1-1). Alternatively, the apoptotic signal can be delivered via the interaction of FasL or TNF-related apoptosis-inducing ligand (TRAIL) on the surface of the NK cell with their respective ligands present on target cells (21, 22). In addition to promoting the induction of target cell apoptosis, the IS is involved in the release of NK-derived cytokines such as interferon gamma (IFN- γ), and tumor necrosis factor alpha (TNF- α) (23). The release of NK-derived IFN- γ can induce antigen presenting cells activation and, in turn augment antigen presenting cell phagocytic and MHC antigen processing capacities, thereby modulating the MHC-specific cell mediated response. In addition, the NK cell secretion of TNF- α and chemokines act as pro-inflammatory stimuli which promote the recruitment and extravasation of inflammatory cells at the site of infection. Together, NK-derived cytokines can skew the immune response to favor the clearance of the viral infection (24, 25).

C. NK Cells and Hybrid Resistance

One of the earliest observations that provided some insight into NK cell recognition was the phenomenon known as hybrid resistance (HR). It referred to the ability of a lethally irradiated F₁ mouse to reject bone marrow grafts from either parent (26). This observation contrasted with the accepted transplantation

paradigm at the time, where solid organ grafts or skin grafts were solely rejected based on non-self determinants on the graft. During the quest to understand the origin of the cells mediating HR, it was determined that cells derived from the bone marrow, or "M cells", were responsible for this phenomenon (27). Subsequently, it was determined that NK cells were the cells responsible for mediating HR as it was still evident in severe combined immuno-deficiency (SCID) mice which have a severely compromised B and T cell compartment (28, 29). The ability of NK cells to mediate HR was not reconciled with classical transplantation until MHC I was determined to regulate NK cell recognition by a concept proposed by Kärre and colleagues in what came to be known as the "Missing Self Hypothesis".

D. Major Histocompatibility Complex

In order to discuss "missing self" and attempt to understand NK cell regulation, it is necessary to understand and review the MHC complex. The MHC gene cluster is located on mouse chromosome 17, or human chromosome 6. In mice, they are known as *H-2* genes; while they are referred to as Human Leukocyte Antigens (HLA) in humans (Table 1-1). MHC genes are grouped into three categories: class I, class II or class III where each class represents a different function in immunity. MHC III genes include cytokines (TNF- α and TNF- β), complement proteins (C2 and C4) and some proteins involved in antigen processing (Lmp2, and TAP). MHC I and II proteins, on the other hand, are a group of highly polymorphic genes that present antigens in the form of peptides that can be recognized by CD4⁺ and CD8⁺ T cell subsets, respectively, through their respective TCRs. MHC II proteins are only expressed on the surfaces of antigen presenting cells, whereas almost all nucleated cells express MHC I proteins. MHC II proteins display peptides formed as a result of the phagocytosis and breakdown of pathogenic organisms by antigen presenting cells, and present these peptides to CD4⁺ T cells. On the other hand, MHC I proteins bind to peptides that are derived from the degradation of intracellular cellular and viral proteins which in turn can be recognized by CD8⁺ T cells. In other words, MHC I

proteins provide a means for the immune system to monitor intracellular infections, and the overall health of the cell.

MHC I proteins are non-covalently associated heterodimers comprised of an N-linked glycosylated 44-47 kilo Dalton (kDa) polypeptide (heavy chain, HC), and a smaller 12 kDa beta 2 microglobulin ($\beta 2m$) light chain (Fig. 1-2). Co-association of the two polypeptides is required for the stable surface expression of mature MHC I. For instance, cells from mice deficient in $\beta 2m$ do not express surface HC (30). In addition, mutant cell lines lacking $\beta 2m$ obtain surface MHC I expression upon the ectopic transfection of only the $\beta 2m$ gene (31). The HC contains three extracellular Immunoglobulin (Ig) domains: $\alpha 1$, $\alpha 2$, and $\alpha 3$; and a single transmembrane region that spans the membrane into the cytosol. $\beta 2m$ non-covalently associates with the $\alpha 3$ domain, while the MHC I peptide binding capacity is fulfilled by the $\alpha 1$ and $\alpha 2$ domains. The tertiary structure of these two domains forms two alpha helices (one from each domain) making a groove that can bind peptides. The size of this cleft limits the bound peptides to 8-10 amino acid residues in length (32).

E. Missing Self Recognition – The Missing Self Hypothesis

How can NK cells distinguish between normal cells and virally infected, or tumor cells? In fact, how are healthy cells protected from NK autoaggression, and why do NK cells reject both parental grafts in an F_1 hybrid mouse? In 1986, Klas Kärre and colleagues proposed an idea suggesting that NK cytotoxicity of altered cells stems from their loss or absence of MHC I, rather than from any other changes to them (33). Kärre used a series of elegant experiments to demonstrate that NK cell cytotoxicity was inversely proportional to the presence of MHC I on target cells. He selected for mutant tumour cells that expressed lower levels of MHC I by eliminating MHC I^{high} cells using specific antibodies and complement, then injected either the parental or mutant tumor cells into syngeneic mice (33-35). Only the mutant tumor cells did not grow in the mice, suggesting that the absence of self-MHC I on the tumor cells conferred a growth disadvantage *in vivo*. To link this observation to NK cells, he depleted NK cells

by injecting mice with anti-asialo-GM1, which resulted in increased tumor growth. In other words, NK cell recognition was limiting the MHC deficient tumor's spread because it was susceptible to NK-mediated cytotoxicity, but upon the re-introduction of MHC I, NK control of the tumour was lost, and the tumour could overwhelm its host in the absence of both a T and an NK cell response.

Kärre proposed two models to describe how MHC I was quelling NK cell cytotoxic responses: the "target interference", or the "effector inhibition" model (36). In the former model, a large density of MHC I molecules actively hindered an NK activating receptor from engaging its ligand on the target cell, whereas the latter one proposed that MHC I on the target cell delivered a negative signal that inhibited NK cell cytotoxicity. Direct evidence supporting Kärre's effector inhibition model was first demonstrated with the observation that NK-sensitive target cells expressing the MHC I molecule H-2D^d are completely resistant to IL-2 activated mouse NK cells expressing the inhibitory receptor Ly-49A (37). Later, it was established that H-2D^d was directly recognized by Ly-49A thereby firmly establishing a receptor/ligand relationship between Ly-49A and a MHC I molecule (38). Similarly, the inhibitory receptor, KIR2DL1, was shown to deliver an inhibitory signal on human NK cells following recognition of its HLA-C ligand (39). It is now evident that NK cells in both mouse and humans express a large repertoire of inhibitory receptors which can engage MHC I and deliver an inhibitory signal. Upon the introduction and corroboration of Kärre's hypothesis, HR of bone marrow grafts can be properly explained. According to the Missing Self Hypothesis, NK cells will engage and eliminate cells with a "missing self", or cells originating from either parent. However, NK cells are not uniform in their expression of cell surface receptors. Instead, they are heterogeneous in their expression of inhibitory receptors which have distinct specificities for MHC I alleles. Consequently, NK cells that do not express an inhibitory receptor recognizing one of the parental MHC I molecules will be free to destroy the grafts (Fig. 1-3).

From studies as early as those establishing from HR, it was evident that NK cells have the capacity to destroy normal bone marrow cells. So how can NK

cells remain inert to normal self cells despite the presence of positive NK cell signals? As highlighted by the Missing Self Hypothesis, MHC I plays an integral role in maintaining NK cell protection of 'unaltered' cells (33). MHC I also plays an important role in regulating NK cell tolerance to self. For example, B6 mice expressing a transgenic MHC I H-2D^d allele (normally just H-2^b) no longer accept bone marrow grafts from normal non-transgenic B6 mice, suggesting that NK cells are only tolerant to cells expressing self-MHC I (40).

These observations can be explained using Kärre's effector inhibition model where an inhibitory receptor recognizing self-MHC I prevents NK autoaggression. Thus, MHC I plays a pivotal role in maintaining self tolerance. However, several properties of the MHC I inhibitory receptor system add several layers of complexity to this process. First, MHC I is highly polymorphic with a large amount of allelic variation. In addition, individual NK inhibitory receptors can only recognize certain molecules or groups of MHC I. For example, Ly-49A recognizes the H-2D^d MHC I allele, whereas another inhibitor - Ly-49C recognizes H-2K^b and both receptors are expressed on subsets of NK cells from a B6 mouse (41). Furthermore, a linked genetic mechanism of inheritance is not possible as MHC I genes are inherited independently from inhibitory receptor genes. Moreover, MHC I-specific inhibitory receptors are only expressed by a subset of NK cells, and their expression is mediated by mostly a random process (41). In other words, many NK cells will express inhibitory receptors that will recognize an MHC I allele presented by the host, but some NK cells may not express inhibitory receptors recognizing self-MHC I.

Refinement of Missing Self - The Requirement of an Activating Signal

The lack of recognition by an inhibitory receptor for MHC I is not sufficient to induce an NK cytolytic response - an additional positive signal by an activating or stimulatory receptor is required. In other words, a positive signal generated from an activating receptor can initiate and trigger the cytolytic machinery in the absence of inhibitory receptors signals (see Fig. 1-3) (42). Activating receptors that recognize viral or "altered self" protein products provide

this positive signal for cytolysis (42). For example, the triggering receptor Ly-49H substantially augments NK activity by recognizing the MCMV MHC-like m157 gene product on virally infected cells (43). In addition to activating receptors recognizing specific 'altered' self ligands, NK cells express natural cytotoxicity receptors (NCR), such as NKp30, NKp46 and NKp44, which provide them with the means to 'naturally' lyse tumor cells (44). Together, these receptors confer NK cells with natural cytotoxicity capacities because these receptors collectively sense for the expression of altered self ligands, and cumulatively provide NK cells with positive signals that promote cytotoxicity. However, receptors that only provide positive cytotoxic signals in the presence of signals derived from the adaptive immune response (e.g. CD16 engagement of IgG) are not considered to contribute to natural cytotoxicity. Thus, the outcome resulting from the engagement of an NK cell with its target is a direct reflection of the combined input of positive and negative signals from activating, and inhibitory receptors.

Several mechanisms have been proposed by different groups to explain how NK cells maintain a tolerance to self, and none of these mechanisms are mutually exclusive. The 'at least one' model is in line with the Käre's effector inhibition model where it explains NK tolerance to self by suggesting that 'at least one' NK inhibitory receptor recognizing self-MHC I is expressed on the surface of every NK cell. In this process, maturing NK cells sequentially express different inhibitory receptors until the cell expresses one or more inhibitory receptors for self. Indirect evidence for this model was initially provided by the observation that in human NK clones derived from two different donors, each clone expressed an inhibitory receptor specific for the donor's MHC I molecules (45). Other studies suggested that NK cells 'acquire' the expression of inhibitory receptors in an MHC I-dependent manner (46). In contrast, NK cells purified from a $\beta 2m$ deficient animal were also tolerant to self splenic blasts *in vitro* and *in vivo* – suggesting that MHC I is not the only player in conferring tolerance (47, 48). Interestingly, the cells derived from this deficient mouse exhibited reduced NK effector responses to tumor cells.

The importance of MHC I in promoting NK cell cytolytic capacity was corroborated from other studies (49, 50). Work from David Raulet's laboratory clearly demonstrated that nearly 10 % of splenic NK cells lacked the expression of all the inhibitory receptors known to interact with self-MHC I in a B6 mouse. It also suggested that this NK cell subset had a reduced capacity to lyse NK-sensitive tumor targets, or normal cells lacking the expression of MHC I. These NK cells were also less able to trigger an effective cytokine, or cytolytic response following the crosslinking of several activating receptors, and this difference did not stem from a difference in receptor expression. Similarly, Wayne Yokoyama's group provided more insight into this phenomenon by engineering a mouse that only expressed H-2K^b, the ligand for Ly-49C, or a mouse that only expresses H-2D^d, the ligand for Ly-49A. Ly-49C⁺ NK cells derived from the H-2K^b mouse were more functionally potent than their Ly-49C⁻ counterparts. Likewise, the Ly-49A⁺ NK cells from an H-2D^d mouse were also more cytolytic than the Ly-49A⁻ NK cells (49). The authors proposed that self-MHC I-specific inhibitory receptors expressed on the surface of NK provided these NK cells with positive signals that "licensed" their effector functions; however, the nature of these signals remain to be defined (49). Thus, inhibitory receptors' interactions with self MHC I impart NK cells with a higher functional capacity through a mechanism independent from the expression of activating receptor genes, or with proteins required for NK-mediated cytotoxicity, that involves either 'disarming' NK cells (according to David Raulet), or 'licensing' them for cytotoxicity during their development (according to Wayne Yokoyama) (49, 50) (Fig 1-4).

F. Mouse NK development

NK cell development occurs mostly in the bone marrow and can be separated into three stages (Fig. 1-5): the generation of committed immature NK precursors (iNK) from hematopoietic stem cell progenitors (HSC), the acquisition of receptors involved in target cell recognition, and the final development into fully functional mature NK (mNK) cells. In the first phase, HSC commit to produce iNK cells, thereby preventing further differentiation into erythroid or

myeloid precursors. At the end of this phase, CD122 (interleukin-2 receptor beta (IL-2R β)) is induced. iNKs are non-cytolytic cells expressing the surface markers CD122⁺/NK1.1⁺/CD69⁺/NKG2A^{high} (51). Although they require several cytokines during their maturation, IL-15 is the only one that is clearly indispensable for the development of mature NK (mNK) cells.

iNK cells express the IL-15 receptor complex comprised of CD122 and CD132 (γ_c), and this expression is important for their survival (52, 53). Mice deficient in either receptor, the unique IL-15 receptor α (IL-15R α) expressed by bone marrow stromal cells and antigen presenting cells, or in IL-15 do not develop mature NK cells (54, 55). Unlike other cytokines, IL-15 does not function as a soluble factor. Instead, following its synthesis, it binds to its unique high affinity IL-15R α receptor expressed by the same cell, and engages NK or memory CD8⁺ T cells expressing its receptor complex (56). Developing iNKs receive IL-15 survival signals from bone marrow stromal cells expressing IL-15R α (57, 58). It is also highly likely that such IL-15 trans-presentation provides peripheral mNK with survival signals (59).

The transition into the final stage of NK development is marked by the uniform expression of DX5 and CD122 (60). During this stage, they acquire their tolerance for self-MHC I and their cytolytic capacities. Functionally, cells expressing uniform levels of these markers have a high cytotoxic capacity and express inhibitory receptors recognizing self-MHC I. Once activated, they can enter the periphery where they can fulfill their roles in immunosurveillance, the release of cytokines, or the 'priming' of the adaptive immune response.

G. The NK Complex

Mice contain a 4 Mb genomic region on the distal portion of chromosome 6 known as the NK complex (NKC) (Fig. 1-6). This region encodes genes important for NK cell function, and is highly enriched in DNA sequences corresponding to activating and inhibitory NK C-type lectin receptors (61). These genes are clustered in a region that is flanked by the alpha-2-microglobulin ($\alpha 2m$) gene, and the proline-rich protein (Prp) gene cluster (62, 63). Human NK cells

also express C-type lectin genes encoded in their NKC found on human chromosome 12 (61).

The importance of the NKC in immune function has been highlighted from mouse genetic studies that have mapped genes responsible for viral immunity, or immune function to regions within the complex. For example, the mouse *Cmv1* locus confers resistance to MCMV; whereas resistance to ectromelia virus is mapped to a different region in the the NKC (14, 64). Similarly, the *Chok* gene responsible for conferring murine NK cells with the ability to destroy chinese hamster ovary tumor cells was also mapped to that region (65). Later, both the *Cmv1* and the *Chok* loci were identified as members of the Ly-49 receptor family (65, 66).

In addition to the Ly-49 receptor family, two additional NKC gene cluster families are expressed on mouse and human NK cells: the NKRP-1 family from which NKRP-1C or NK1.1 has extensively been used to characterize NK cells from a B6 mouse, and the NKG2(A-H)/CD94 family of hetero- and homodimers. Both of these families encode activating or inhibitory receptors, and are not restricted in expression to NK cells. Inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIM, consensus sequence I/V/L/SxYxxL/V (42)) within their cytoplasmic tail which trigger a biochemical signaling cascade leading to the suppression of NK cytotoxicity or cytokine release. Activating receptors lack ITIMs, or intrinsic signaling capacities. Instead, they associate with signaling adapter proteins such as DAP12, DAP10, or Fcγ through intramembrane electrostatic interactions. These adapters contain immunoreceptor tyrosine-based activation motifs (ITAM, consensus sequence D/ExxYxxL(x)₆₋₈YxxL/I (42)) or other motifs that trigger NK effector functions (Fig. 1-7).

H. NK receptors of MHC I

Because NK cells express many receptors that modulate their activities, only those relevant to this thesis will be introduced. The KIR MHC receptors will be discussed in addition to the mouse NK receptors because some of the conclusions

drawn from this thesis will be extended to human NK cells in the *Discussion* chapter.

KIR and Ly-49 – different receptors, similar functions

The killer cell immunoglobulin-like receptor (KIR) and the Ly-49 family of receptors are likely to be examples of convergent evolution. Like the *Ly-49* family, *KIR* is a large multigene family of MHC I receptors expressed on human NK cells, NKT cells, and some CD8⁺ T cells. Unlike *Ly-49*, *KIR* are not expressed by NK cells in rodents and are not a part of the NKC. Instead, they are found as a cluster of genes in a region known as the leukocyte receptor complex (LRC) present on chromosomes 19q26 or 7 in humans or mice, respectively. Certain *KIR* haplotypes are phenotypically associated with innate or autoimmune responses such as the elimination of tumors, rheumatoid arthritis or psoriasis in humans (67-69).

KIR and Ly-49 are functional orthologues, but are structurally distinct. Ly-49 are C-type lectins that are type II membrane receptors expressed as disulphide-linked homodimers on the cell surface (67). Each monomer of an inhibitory Ly-49 contains a single ITIM within its cytoplasmic tail. KIR, on the other hand, are type I transmembrane receptors with two or three immunoglobulin-like (Ig-like) domains in the extracellular domain, and long (containing two ITIMs) or short cytoplasmic tails, corresponding to inhibitory or activating receptors, respectively. 'Short' KIR are truncated forms of full length receptors, and thereby lack ITIMs, but contain the transmembrane charged residue required for DAP12 association (70). The KIR extracellular Ig domains dictate the HLA allele specificity, while their intracellular domains assign their functional capacities. Importantly, the inheritance of activating KIR genes encoding a similar extracellular domain as an inhibitory receptor gene is believed to stem from gene duplication, followed by recombination events (71). An example of such an event is illustrated by the gene pair *KIR2DL1* and *KIR2DS1* (where the former has the long tail, and the latter has the shorter one). Studies using the extracellular domain of KIR2DL1 and KIR2DS1 suggest that inhibitory

KIR bind with a higher affinity than activating KIR to their HLA-Cw4 natural ligand (72). Thus, it is possible that activating KIR have evolved to monitor viral infection through minor alterations in HLA structure (73).

Ly-49 Receptors

The *Ly-49* gene family, like *KIR*, are a multigene (*Ly-49A-X*) receptor family (41, 74) that unlike *KIR*, are clustered in the most telomeric region in the NKC on mouse chromosome 6 (62). With the exception of *Ly49B*, all *Ly49* genes are organized in tandem within a region 420 kb in length (75). In addition, Southern blot, RT-PCR and flow cytometric analyses from B6, 129/J, BALB/c, and non-obese diabetic (NOD) mouse strains have demonstrated that different inbred mouse strains have different *Ly49* gene content and receptor expression profiles (62, 74, 76-78) (Table 1-2). In addition to differences in gene content, different strains also encode different alleles of the same gene – also contributing to *Ly-49* receptor diversity between mouse strains. For example, NK cells derived from B6 and BALB/c strains both express *Ly-49G*; however, the two strains express different alleles of this receptor which can be discriminated by different antibodies (79, 80).

Ly-49 are expressed on the surfaces of murine NK cells, NKT cells, CD8⁺ T cells, macrophages (for *Ly-49B*) and plasmacytoid dendritic cells (pDC, for *Ly-49Q*) (41, 81, 82). Like *KIR*, there are two types of *Ly-49* – activating and inhibitory. Interestingly, like *KIR* they act as receptors for MHC I and virally encoded MHC I-like molecules in some cases, although the recognition of MHC-like viral ligands by *KIR* remains to be defined. NK cell expression of individual *Ly-49* receptors is heterogeneous; i.e. an individual NK cell can express several *Ly-49* receptors simultaneously on its surface (83-85). In fact, it has been suggested that the frequency of an NK cell expressing different *Ly-49* receptors is governed by the “product rule” where the probability for expressing two receptors simultaneously is the product of their respective frequencies. Furthermore, the acquisition of inhibitory *Ly-49* expression by NK cells occurs through a stochastic process (86). On the other hand, activating *Ly-49* induction is more

tightly regulated. Studies using NK cells derived from a B6 mouse have shown that NK cells expressing the activating receptor Ly-49H preferentially also express Ly-49D (87). Moreover, Ly-49 receptors are expressed mostly in a monoallelic fashion, although a minority of NK cells express a receptor from both alleles (88, 89).

The variegated expression of Ly-49 is primarily controlled at the transcriptional level (90). In fact, a splenic mNK cell can express from zero to five different Ly-49 receptors, with an average of two or three receptors per cell, as determined by single cell RT-PCR. Studies conducted by Saleh and colleagues have provided an exquisite mechanism describing how Ly-49 receptor transcription is regulated (91). The model involves a distal bidirectional promoter, Pro1, proximal to each *Ly-49* gene only active in iNK cells, that is followed by two distal promoters active in mNK cells. In the active *Ly-49* allele, activation by competing transcription factors at the Pro1 site in the forward direction favors transcription at the other distal *Ly-49* promoters, while transcription in the reverse direction at the Pro1 site inhibits the transcriptional activation at the distal *Ly-49* promoters. Once forward transcription occurs at the Pro1 site for *Ly-49* in an immature NK cell, it is maintained for that *Ly-49* throughout development and in the mNK cell. Upon expression, the interactions between Ly-49 and self-MHC I play a critical role in maintaining tolerance to self.

Ly-49 receptors recognize MHC I in an allele specific manner (37, 92, 93). Recognition of MHC I is mediated by the Ly-49 carbohydrate recognition domain (CRD) in the extracellular domain (94). Interestingly, Ly-49 stalk regions (~70 aa) are substantially longer than similar regions in other receptors – suggesting that they may recognize MHC I present on the same cell (*cis* recognition) as well as MHC I on different cells (95). In fact, this mechanism of *cis* recognition has been proposed as a method in regulating cellular responses to ligands expressed on other cells (96, 97).

Many of the models developed to explain the mechanism regulating Ly-49 specificity for MHC I have been based on the co-crystal structure proposed by

Tormo and colleagues between the CRD region of the inhibitory Ly-49A and its H-2D^d MHC I ligand (98). In this co-crystal, two potential interaction sites between Ly-49A and H-2D^d were suggested. The first, or 'site 1' interaction, is located at one end of the peptide-binding groove in the $\alpha 2$ domain of the MHC I heavy chain, while the other, or 'site 2', interaction is present under the more conserved peptide-binding region comprising areas in the $\alpha 3$ region of the heavy chain and residues within $\beta 2m$. It is now clear that site 2 is the primary interaction site as the mutagenesis of single solvent-exposed residues in this region abrogated Ly-49 recognition (99, 100). So how does Ly-49 maintain its allele specificity when it interacts with a region of MHC I that is highly conserved? Very recently, it has been suggested that Ly-49 receptors have a preference for MHC I supertypes, or groups of MHC I allele products that share a preference for specific amino acid residues within the bound peptide. Remarkably, mouse Ly-49 receptors recognizing specific mouse MHC I alleles also interact with rat MHC I sharing the same supertypes as the mouse MHC I alleles they recognize. These studies suggest that supertype recognition may be a fundamental function of Ly-49 receptors (101).

Activating Ly-49

Activating Ly-49 receptors, like their inhibitory counterparts, recognize MHC I or viral homologues of MHC I. For example, Nakamura and colleagues first demonstrated that Ly-49D, an activating receptor expressed in B6, 129/J and NOD mouse strains, recognizes H-2D^d, but not another MHC I allele H-2D^b (102). Similarly, Ly-49P, another activating receptor expressed in the NOD strain, also recognizes H-2D^d but not H-2D^b and also weakly interacts with H-2D^k (103). Structurally, activating and inhibitory Ly-49 are highly homologous within their extracellular domain – suggesting the presence of paired receptors similar to the KIR receptor system in humans. Consequently, recognition by activating Ly-49 of self MHC I molecules may represent a mechanism, as possibly with activating KIR, for detecting 'altered' self during immunosurveillance.

Activating Ly-49 receptors do not contain ITIMs in their cytoplasmic tails. Instead, they associate with the adapter DAP12 through an electrostatic interaction within the plasma membrane (104). DAP12 is a disulphide-linked homodimer of 12-15 kDa containing a single ITAM within its cytoplasmic tail (70). Upon tyrosine phosphorylation of its ITAM, it preferentially recruits members of the splenocyte tyrosine kinase (Syk) family (105). DAP12 is expressed in NK cells, macrophages, myeloid-derived DCs, granulocytes, mast cells, basophils and osteoclasts (106). The DAP12 association is both important for activating Ly-49 functions and for their surface expression. These relationships are highlighted from studies using DAP12 deficient mice which have reduced levels of cell surface activating Ly-49D and H – the only activating receptors present in the B6 mouse (107). Also, NK cells expressing an ITAM^{null} form of DAP12 do not efficiently lyse P815 cells in a redirected lysis assay following anti-Ly-49D antibody treatment (108).

Activating Ly-49 receptors trigger NK cell cytotoxicity and cytokine release following the engagement of their ligands, in a DAP12-dependent manner. *Cmv1*, the locus conferring resistance to MCMV in certain mouse strains, was originally mapped to the NKC (109). Subsequently, Ly49H was identified as the receptor responsible for immunity from this locus (66, 110). In fact, Ly-49H directly recognizes the MCMV MHC-like m157 gene product (43). Similarly, Ly-49P protects the Ly-49H negative MCMV-resistant Ma/My mouse strain via its interaction with H-2D^k (111). Interestingly, the Ly-49P interaction with H-2D^k only occurs in the context of MCMV infection (111). However, the molecular mechanism of this interaction still remains to be defined (112).

I. Non-MHC I NK Activating Receptors

In addition to the Ly-49/MHC I receptor system, mouse NK cells express a variety of activating receptors not specific for MHC I which also associate non-covalently with signaling adapters. For the purpose of this thesis, only NKG2D, NKRP-1 receptors, and CD16 will be discussed.

NKG2D – an activating receptor of mouse and man

NKG2D is an activating receptor expressed by NK cells of both mice and humans. It acts as an activating receptor on NK cells, and is a co-receptor for activated CD8⁺ T cells (113). Like activating Ly-49, it is encoded by a gene in the NKC, is a type II membrane receptor of the C-type lectin family, and is a homodimer. In contrast to Ly-49, NKG2D is a single copy receptor that is conserved in both species.

When it was cloned, NKG2D was identified as a receptor that directly recognized the MHC-like MIC family of proteins which are induced by cellular stress (114). NKG2D in human recognizes members of the MHC I-like MIC family (MIC-A, B, C), and members of the UL-16 binding proteins (ULBP) which, as suggested by the name, bind the HCMV UL16 protein (115). In mice, NKG2D recognizes members of the retinoic acid early transcripts (α , β , γ , δ), H60, or the mouse ULBP homologue Mult1 which are acquired in expression as a result of transformation, or cellular activation (116, 117). Interestingly, NKG2D ligand expression is induced on cells upon the induction of the DNA damage response, thus the NKG2D receptor/ligand system can provide positive signals that culminate in the elimination of tumor cells by NK and CD8⁺ T cells (118).

NKG2D is constitutively expressed on the surface of most mouse and human NK cells. It is also expressed on most NKT cells, some γ/δ T cell subsets and is inducible in expression on the surfaces of CD8⁺ T cells in both species. In humans, a single form of NKG2D is present which associates with the adapter DAP10/KAP10 via an electrostatic intra-membrane interaction. Like DAP12, DAP10 is encoded in the LRC in both mice and humans (119). In contrast, DAP10 does not contain an ITAM within its cytoplasmic tail. Instead, it contains a YxNM motif which recruits members of the phosphoinositide 3-kinase (PI3K) family of enzymes following recognition (120).

In mice, NKG2D is present as either a full-length long form (NKG2D-L) or a shorter form (NKG2D-S) as a result of alternate splicing. NKG2D-L is expressed only in unstimulated NK cells, and preferentially associates with DAP10, whereas NKG2D-S is expressed in IFN α/β -activated NK cells and in

activated CD8⁺ T cells (113, 121). NKG2D-S can associate with either DAP10 or DAP12; however, because CD8⁺ T cells do not express DAP12, NKG2D-S expressed in T cells only associates with DAP10 (113, 121). The preferential association with DAP10 stems from an additional 13 amino acid N-terminal sequence in NKG2D-L which prevents NKG2D association with DAP12 (122).

NKRP-1 – the prototype for MHC-independent self/non-self discrimination

As previously indicated, the NKRP-1 family of receptors is also present in the NKC region. NKRP-1 family receptors are C-type lectins that are type II homodimeric membrane glycoproteins. This family includes the activating receptor NKRP-1C (NK1.1) (123) originally identified as a marker for NK and NKT cells in B6 mice (124, 125). Other members include the activating receptor NKRP-1F, which like other activating receptors, contains a positively charged transmembrane residue, and the ITIM-containing inhibitor NKRP-1D.

NKRP-1 family members, like Ly-49 receptors, do not require coordinate binding of calcium for their interactions with their ligands. Known ligands for NKRP-1 family members are not MHC or MHC-like proteins. Instead, NKC encoded C-type lectin related (Clr) B and G are ligands for the inhibitory NKRP-1B and activating NKRP-1F, respectively (126). Very recently, Voigt and colleagues demonstrated that rat CMV evades NK cell destruction of infected cells by encoding a decoy "self" ligand for the inhibitory NKRP-B receptor (127).

The ligand for NKRP-1C has not been clearly defined. However, the mechanism by which it triggers activation resembles other immune-activating receptors, as it associates through an electrostatic interaction with the ITAM containing adapter protein FcR- γ (128). This adapter is very similar in structure and highly homologous in sequence to DAP12, as it contains a single ITAM within its cytoplasmic tail, is expressed as a homodimer, and has a short extracellular domain with a transmembrane domain containing a negative charge. Fc γ is also the adapter employed by the low-affinity IgG receptor CD16 (Fc γ RIII) to trigger NK effector functions such as antibody-dependent cellular cytotoxicity (ADCC), or cytokine release.

CD16 – linking innate cells to the humoral response

CD16 (FcγRIII) is a member of the Ig-superfamily of receptors that binds antigen-antibody immune complexes (IC) with a low affinity (K_d 2×10^7 M⁻¹) (129). Human CD16 is either a transmembrane receptor (FcγRIIIa) that electrostatically associates with FcR-γ and/or CD3ζ, or is in glycerophosphatidyl inositol (GPI) linked form (FcγRIIIb). Human FcγRIIIa is expressed on macrophages (130), NK cells (131), and γ/δ T cells (132), whereas FcγRIIIb is predominantly expressed in neutrophils (129). In contrast, mouse FcγRIII is only expressed as a transmembrane receptor in NK cells, macrophages, neutrophils, and mast cells (129, 133). Moreover, mouse NK cells do not express CD32, an inhibitory low affinity IC receptor highly homologous to FcγRIII. Following stimulation of NK cells through CD16, NK cells are triggered to mobilize and secrete their cytotoxic granule contents toward the target cell, in a process known as ADCC. Alternatively, the same stimulus can induce NK cells to release IFN-γ (134). Release of intracellular Ca⁺⁺ stores, phosphoinositide turnover and elevated protein kinase activity are signaling events in common in CD16 stimulated ADCC and cytokine secretion by NK cells (134, 135).

J. NK signaling and adhesion

As previously discussed, NK cell responses are governed by a combined input of activating and inhibitory signals. Because NK cells are equipped with an arsenal of cytolytic mechanisms and cytokines, the signals they receive within their microenvironment must be tightly regulated. These signals include the interaction of inhibitory and activating receptors with their ligands. Following ligand recognition by these receptors, a number of biochemical signaling cascades are initiated which either favor NK effector functions, or favor the quelling of these mechanisms.

When challenged with a normal cell, an NK cell maintains its tolerance to self because inhibitory signaling events dominate the outcome within the NK cell. These signaling events require the activity of ITIMs within inhibitory receptors engaging their ligands, and tyrosine phosphatases which extinguish positive

signals that would otherwise trigger the NK cell. Upon ligand binding, tyrosine residues are phosphorylated, probably by a Src family kinase (SFK). This promotes the recruitment of SH2 containing phosphatase-1 (SHP-1) or SHP-2 tyrosine phosphatases, or SH2 containing inositol phosphatase (SHIP) phosphatidylinositol phosphatase via their SH2 domain (136). For KIR or Ly-49 receptors, it is established that SHP-1 is recruited to the phosphorylated ITIM as it co-immunoprecipitates with the receptors (137-139). Upon recruitment, SHP-1 acts on membrane proximal targets, dampening the activation cascade. Because these signaling targets are relatively upstream in the activation cascade, the inhibitory signal can terminate activation signals prior to their amplification.

The precise molecular targets recognized by SHP-1 have not been fully defined; however, several studies have implicated SHP-1 in regulating molecules involved in remodeling the actin cytoskeleton, or in tight adhesion between NK cells and their targets. For example, using a chimeric receptor comprised of the extracellular domain of KIR2DL1 and the intracellular domain of the phosphatase catalytic domain for SHP-1, tight adhesion between NK cells expressing this receptor and target cells expressing the ligand for the KIR is disrupted (140). A similar observation was evident when rat NK leukemia cells (RNK-16) stably expressing Ly-49A were incubated with susceptible rat cells expressing mouse H-2D^d (**Appendix 1**). Subsequently, in an attempt to identify SHP-1 substrates, Chris Stebbins and colleagues modified the KIR-SHP-1 chimeric receptor to introduce a trapping mutation within the catalytic site (141), and identified the guanine exchange factor Vav-1, involved in cytoskeletal remodeling, as a substrate (142). Together, these studies highlight how NK cells regulate their functional responses by controlling mechanisms facilitating cytoskeletal remodeling, and in turn, tight adhesion.

The Vav family of actin modeling proteins, along with many cytosolic enzymes and adapter molecules play an integral role in regulating NK cell adhesion to target cells, and effector functions. Like inhibitory receptor signaling, the instigation of activating receptor biochemical signaling cascade occurs following ligand recognition. As previously discussed, activating NK receptors

mediate their effector functions by associating with signaling adapters. The molecules involved in both of these signaling processes are highlighted in **Fig. 1-8**, among them SFKs and Syk kinases are important for ITAM signaling, while PI3K signaling is important for DAP10 signaling (42). Although PI3K activity is directly modulated by the recruitment of the regulatory p85 subunit to DAP10, it is also required for DAP12 signaling through a Syk-dependent mechanism as specific inhibitors for PI3K activity inhibit DAP12-dependent cytotoxicity in NK cells, and NK cytotoxicity inhibited by piceatannol, a specific Syk kinase inhibitor, is restored in the presence of a constitutively active form of PI3K (143).

Following ligand recognition of activating Ly-49, KIR, NKRP-1C, or NKG2C receptors, SFKs (ex. p56^{Lck}, p59^{Fyn}) phosphorylate tyrosine residues on the ITAM-bearing adapters. SFK activation requires the dephosphorylation of the regulatory C-terminal tyrosine (Tyr⁵⁰⁸ in p56^{Lck}) by phosphatases such as CD45 (144). Following the dephosphorylation at this position, an autophosphorylation event occurs to impart maximal enzymatic activity to SFKs (145). In IL-2 activated NK cells, the SFK family members p56^{Lck} and p59^{Fyn} co-immunoprecipitate with the Ly-49D/DAP12 receptor complex, and directly phosphorylate DAP12 (146). Moreover, IL-2 activated cells derived from a CD45 deficient mouse, unlike wild type cells, did not release cytokines (IFN- γ) in response to anti-Ly49D, anti-CD16, anti-NK1.1 or anti-NKG2D treatment, suggesting a role for CD45 in regulating SFK (147). Finally, RNK-16 cells expressing the mouse Ly-49P receptor do not lyse cells expressing its H-2D^d ligand in the presence of the SFK competitive inhibitor PP2, as well as piceatannol (**Appendix 2**, unpublished observation).

A requirement for Syk kinase activity for ITAM-dependent cytotoxicity highlights its importance in ITAM-mediated signaling. Following the tyrosine phosphorylation of signaling adapters at the ITAM, Syk family kinases (Syk, and ζ -associated protein of 70 kDa (ZAP-70)) are recruited via their SH2 domains and activated (105). The Syk/ZAP-70 enzymes are not required for NK cell development, as mature NK cells form from bone marrow chimeras deficient in these enzymes. However, Syk family kinases are required for Ly-49/CD16-

dependent cytotoxicity and cytokine release, as redirected lysis through Ly-49D or ADCC is severely reduced (148). Upon recruitment, they phosphorylate a number of signaling molecules, including the SH2 domain containing leukocyte protein of 76kDa (SLP-76), linker of activated T cells (LAT), non-T activated linker (NTAL), phospholipases C γ (PLC- γ), Vav and PI3K which increase intracellular Ca^{++} levels, regulate the cytoskeleton, and induce cytokine release or degranulation (42, 149) (Fig. 1-8, 1-1).

NKG2D/DAP10 signaling is different from ITAM-mediated signaling as the p85 regulatory subunit of PI3K complex is directly recruited to DAP10 following receptor recognition of its ligand. As previously indicated, DAP10 contains a YxNM motif that which was previously shown to recruit the p85 PI3K regulatory subunit with CD28 on T cells (150). The recruited regulatory subunit can attract the catalytic subunit of PI3K, which converts 3'-phosphorylated inositides to PtdIns(3)P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃, that act as switches, which in turn recruit and activate other enzymes within the cell (151). In fact, the NKG2D/DAP10 complex uses PI3K as its major downstream signaling effector molecule (120, 152). Moreover, the DAP10 YxNM motif acts as a docking site for the Grb-2 adapter molecule which bridges NKG2D signals to molecules such as SLP-76 and PLC- γ , thereby amplifying the signal (153, 154).

Tight adhesion is important for Cytotoxicity

Prior to the polarization and exocytosis of cytolytic granules by an NK cell, it must form tight conjugates with the target cell. The formation of these conjugates promotes the stable association of the two cells which is important for the IS, the polarized delivery of granules, and the release of IFN- γ (20). Numerous studies have implicated the integrin LFA-1 (CD11a/CD18, α_L/β_2) as a key player in the tight association between cytotoxic cells and their targets. Integrins like LFA-1 are heterodimers composed of α and β subunits. LFA-1 binds intercellular adhesion molecule-1 (ICAM-1) through ICAM-5, and is expressed on all leukocytes (155-157). NK cells also express lower levels of other integrins (β_1 , CD11b/CD18; CD11c/CD18). LFA-1 is important for NK cell tight

adhesion and cytotoxicity of target cells. For example, NK cells derived from patients deficient in the CD18/ β_2 chain are unable to destroy susceptible targets (158-160). Similarly, IL-2 activated NK cells from mice deficient in CD11a/ α_L are unable to form tight conjugates or eliminate tumor cells (161, 162). Furthermore, anti-LFA-1 antibodies impair human NK clone natural cytotoxicity of tumor cells (163, 164).

Inside-out Signaling

The regulation of integrins, such as LFA-1, by an antigen receptor was first established in T cells by Michael Dustin and Tim Springer (165). Upon the interaction of T cells with APCs expressing the MHC-peptide ternary complex recognized by their TCR, adhesion between the two cells is substantially augmented by upregulating the ability of LFA-1 to engage its ligand ICAM-1. The signaling events which increase the LFA-1 ligand binding capacity, or inside-out signals, transiently increase LFA-1 binding of ICAM-1 (165). LFA-1 increased adhesion to ICAM-1 can either occur through increases in the intrinsic affinity of LFA-1 as it adopts a more open conformation, through changes in LFA-1 lateral mobility, or clustering along the membrane, or a combination of these processes (157). Within these processes, regulators of the actin cytoskeleton appear very important. For example, upon the binding and anchoring of LFA-1 to the actin cytoskeleton by the actin binding protein talin, increased LFA-1 ligand binding is evident. Alternatively, LFA-1 initially away from the region of cell contact can be targeted to this region through the activities of the small GTPase Rap1, and the RapL/Mst 1 complex. These molecules target the recycling of internalized LFA-1 to the region of cell contact, thereby increasing the concentration of LFA-1 available to bind ICAM-1 at the site of cell contact and also facilitate the assembly of the IS and the continuation of antigen-receptor signals (157, 166).

In NK cells, LFA-1 clearly contributes signals that are important for cytotoxicity; however, the manner by which activating NK receptors regulate LFA-1 functions is not defined. Eric Long's laboratory (NIH) has provided some

insight into this process. Using insect cells expressing ligands for different human activating receptors and/or human ICAM-1, they demonstrated that LFA-1 is sufficient for generating biochemical signals that polarize cytolytic granules to the site of cell contact in resting human NK cells (167). Interestingly, granule polarization involves the activation of Rac GTPases by Vav – suggesting a role for LFA-1 in activating Vav rearrangement of the cytoskeleton (168). Degranulation, on the other hand, require signals from LFA-1 and the bonafide activating NK receptor CD16 (167). Moreover, resting human NK cells incubated with mouse P815 FcR⁺ cells in a redirected lysis assay only destroyed target cells when stimulated with CD16, but not NKG2D or other activating receptors. In contrast, when multiple NK receptors were co-engaged, cytotoxic signals were induced. Thus, apparently signals from individual NK receptors synergize to lower the threshold required for degranulation (169).

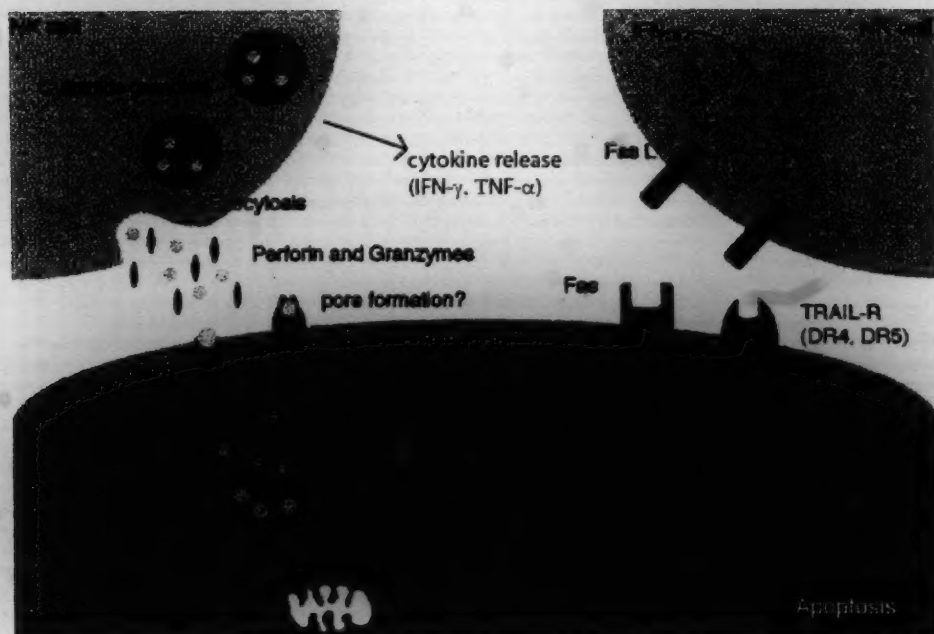
K. Thesis Rationale and Hypotheses

As previously discussed, different mouse strains are heterogenous in their Ly-49 receptor expression, and some strains express different alleles of the same receptor. For example, the BALB/c, B6 and NOD mouse strains express different alleles of the *Ly-49G* inhibitory receptor. These receptors exhibit a high degree of sequence homology; however, certain polymorphisms are evident within their extracellular domains. I hypothesized that regions of polymorphism in *Ly-49G* receptors may be important for MHC I allele specific interactions. Furthermore, several activating *Ly-49* receptors (ex. *Ly-49W* and *Ly-49M*) share a high degree of amino acid identity with inhibitory *Ly-49* receptors within their extracellular domains, and also share the same amino acid polymorphisms present in the different *Ly-49G* receptors. Moreover, the study of individual *Ly-49* receptors has been hampered by the lack of serological reagents that can be used for the detection of related receptors and the discrimination between different alleles of the same receptor. Consequently, we generated two monoclonal antibodies (mAbs), Cwy-3 and CK-1, by injecting NK cells from a B6 mouse into BALB/c which encode different alleles of certain *Ly-49*, including the inhibitory

Ly-49G, with the prediction that these antibodies would recognize *Ly-49* polymorphism(s). I mapped the regions recognized by these two antibodies as two distinct locations on the native *Ly-49G* molecules. The results from these studies are discussed in chapter two of this thesis from the paper entitled "*Epitope mapping of Ly-49G and G-like receptors: CK-1 antibody defines a polymorphic site of functional interaction with class I ligand*" published in the 2005 May issue in the **Journal of Leukocyte Biology** 77(5):644-651.

Tight adhesion is an important step for the cytotoxic destruction of target cells by NK cells and CTL. In fact, as discussed above, the integrin LFA-1 plays a central role in NK cell cytotoxic mechanisms. Studies in T cells have clearly demonstrated that the TCR can regulate LFA-1 binding of ICAM-1, and also tight adhesion, through biochemical signals generated following recognition. These signals require the CD3 complex which associates with the TCR via an intramembrane electrostatic interaction. The CD3 complex is comprised of several signaling adapters that contain ITAM regions within their cytoplasmic tails. Because activating *Ly-49* receptors associate with the ITAM-containing signaling adapter protein, we hypothesized that they too regulate integrin inside-out signaling, but by NK cells. In the third chapter of this thesis, the paper entitled "*Activating Ly-49 receptors regulate LFA-1 mediated adhesion by NK cells*" published in Feb. 2007 in the **Journal of Immunology** is discussed. This paper demonstrated, for the first time, that an NK activating receptor family (*Ly-49* receptors) regulate inside-out activation of LFA-1 in a DAP12 and SFK/Syk-dependent manner. Similarly, chapter four of this thesis extends the hypothesis that regulation of inside-out signaling will include activating the NK receptors CD16, NKG2D, and NKRP-1C. Chapter four describes my results testing this hypothesis.

The final chapter highlights these studies in the broader context of our understanding of NK cell biology. It is also followed by a description of some future studies, in addition to a discussion of studies conducted in chapters 2, 3 and 4, along with those summarized in the *Appendix* section.



Adapted from Smyth MJ *et. al*, *Mol Immunol* (2005) (170).

Figure 1-1. NK cells induce contact-dependent cytotoxicity of target cells through different mechanisms. The granule exocytosis pathway involves the uptake of granzyme (Grz) into target cells may go through a perforin dependent or independent mechanism. Grz then plays a critical role in triggering apoptotic cell death through the activation of cellular caspases, and/or a caspase-independent pathway. Alternatively, members of the TNF family of cytokines, FasL and TRAIL, are expressed by NK cells and regulated by IFN-γ. These molecules can induce a caspase-dependent target cell apoptosis via their corresponding receptors Fas and TRAIL-R (DR4, DR5), respectively. In addition, NK cells can release a variety of cytokines or chemokines (ex. IFN-γ, TNF-α, MIP-1α) which can amplify the immune response.

Mouse MHC Complex (chromosome 17)

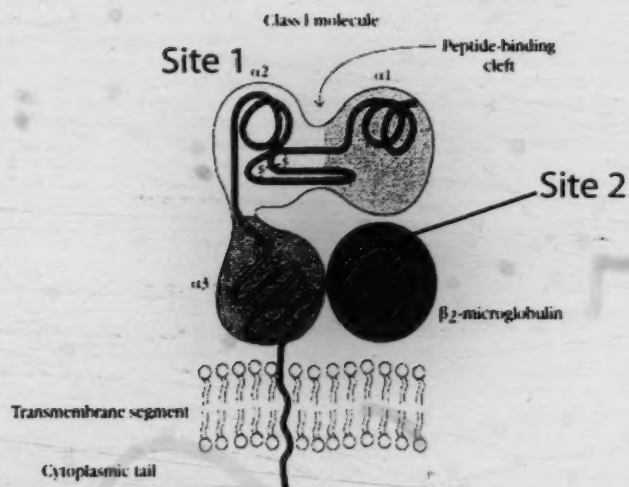
Mouse H-2				
MHC class				
Region				
Gene Product				

Human MHC Complex (chromosome 6)

Human Leukocyte Antigens (HLA)				
MHC class				
Region				
Gene Product				

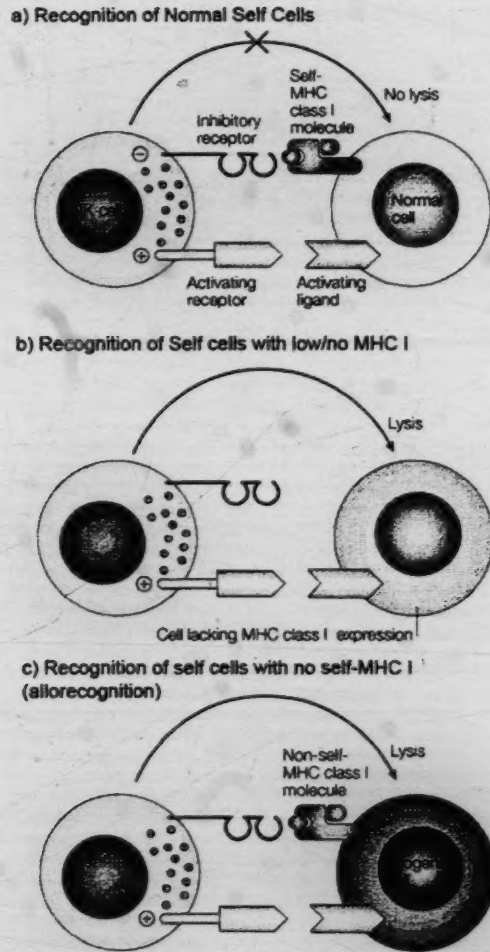
Adapted from **Kuby Immunology** (2000) 4th Edition. Goldsby R, Kindt TJ, and Osborne BA.

Table 1-1. Mouse and Human MHC genes.



Adapted from **Kuby Immunology** (2000) 4th Edition. Goldsby R, Kindt TJ, and Osborne BA.

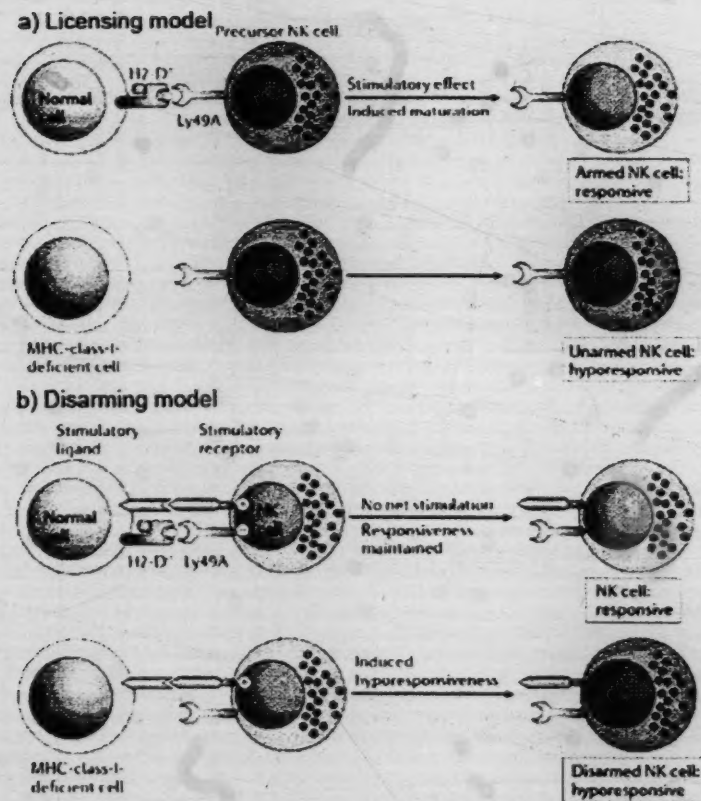
Figure 1-2. Structure of MHC I. The $\alpha 1$ and $\alpha 2$ domains form the peptide-binding cleft, whereas the $\alpha 3$ region interacts non-covalently with $\beta 2m$. The proposed "site 1" and "site 2" regions for Ly-49 recognition are also indicated.



Adapted from Vinay Kumar & Megan E. McNerney *Nat Rev Immunol* (2005) (171).

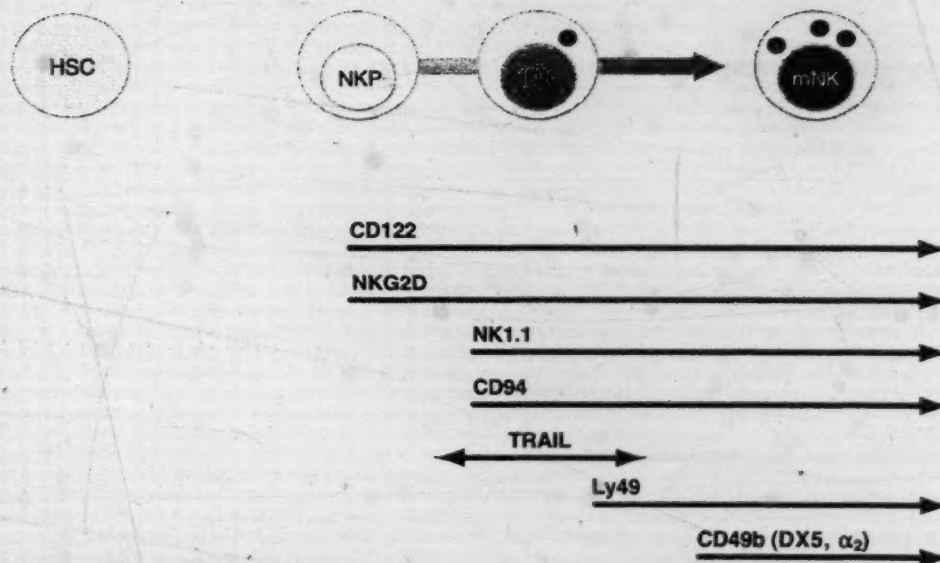
Figure 1-3. Missing Self Recognition by NK cells.

- a) Upon interacting with a normal target cell, an NK cell receiving an activating signal will not destroy the cell because an inhibitory receptor recognizing self-MHC I will inhibit NK cell activation.
- b) If the target cell loses expression of MHC class I molecules, as a result of viral infection or transformation, then inhibitory signal is relieved and the NK cell lyses the target.
- c) Similarly, if the NK cell does not express an inhibitory receptor that can recognize the MHC I alleles present on the target cell, as during HR, the NK cell does not receive an inhibitory signal and destroys the target.



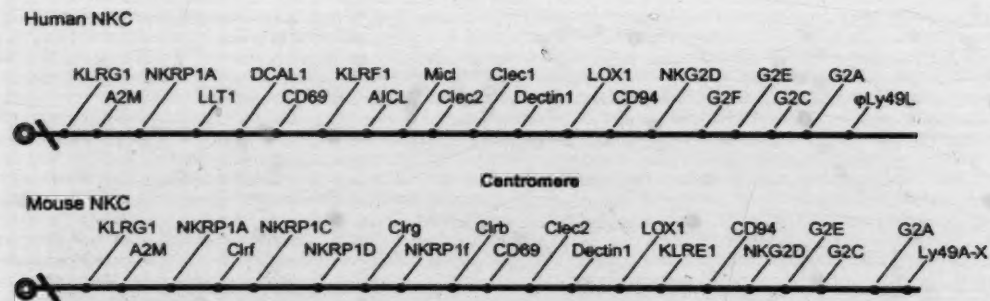
Adapted from Raulet DH and Vance RE *Nat. Rev Immunol* (2006) (172).

Figure 1-4. Licensing vs Disarming of NK cells. A) In the licensing model, maturing NK cells receive positive signals from the interaction of an inhibitory Ly-49 receptor's interaction with stromal cells expressing its MHC I ligand. This interaction is required to induce functional maturation as NK cells that do not receive this interaction remain unlicensed and do not destroy MHC I deficient target cells. B) In the disarming model, NK cells express a variety of stimulatory and inhibitory receptors. By virtue of these receptors, they recognize self cells. However, only maturing NK cells that have balanced inhibitory and activating signals are allowed to retain their responsiveness as cells that do not receive an inhibitory signal have a net positive signal, and are consequently disarmed during development.



Adapted from Di Santo J, *Annu Rev. Immunol.* (2006) (60).

Figure 1-5. Model for NK cell development in the bone marrow. NK cells develop in three stages: generation of NK precursors (NKP), the generation of iNK cells positive for NK1.1, NKG2A, and CD122, and finally the terminal maturation characterized by Ly-49 receptor acquisition, DX5 expression and cytolytic/cytokine releasing abilities.



Adapted from Lanier LL, *Annu. Rev. Immunol.* (2005) (42).

Figure 1-6. The Human and Mouse NKC. The mouse and human NKC share many of the same family members (*NKG2*, *NKRP1*, *KLRG*). However, in the most telomeric region, the mouse NKC has a significant number of *Ly-49* genes whereas the human one has an *Ly-49L* pseudogene.

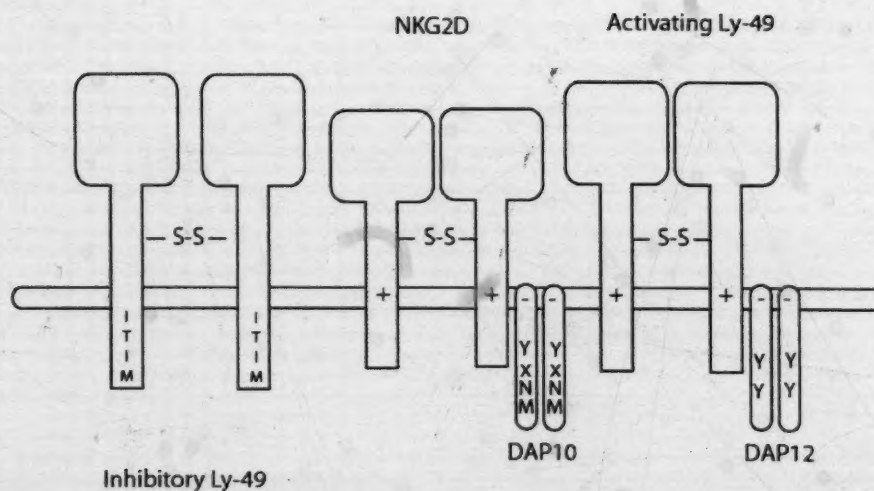
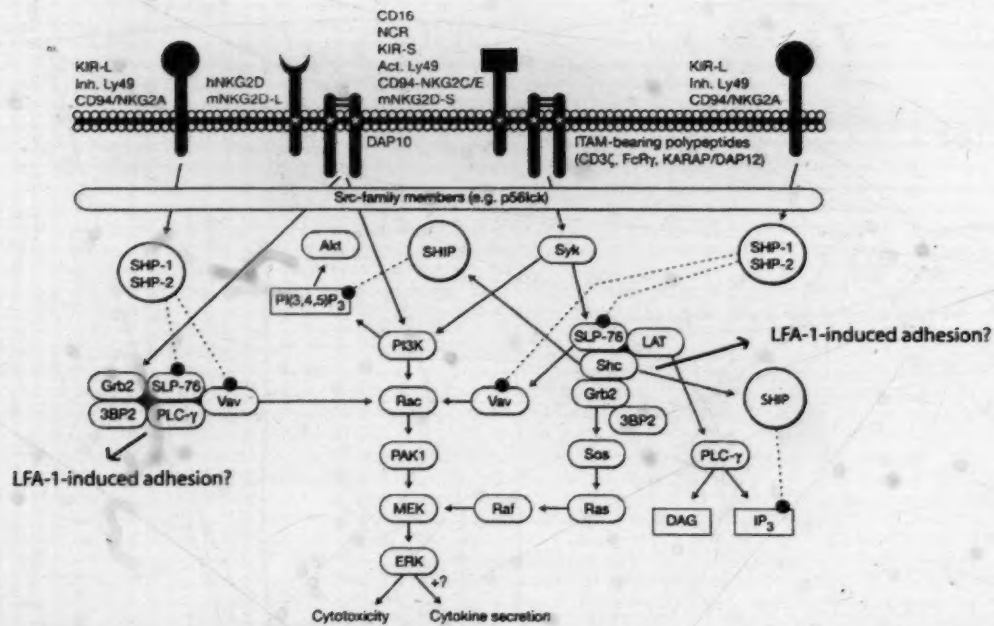


Figure 1-7. Activating and inhibitory NK receptors. Activating receptors signal through their association with ITAM-containing adapter proteins (DAP12, FcR- γ , or CD3 ζ) or the ITAM-free DAP10 (NKG2D only), whereas inhibitory receptors contain ITIMs in their cytoplasmic domains which can directly contribute to signaling.

Strain	C57BL/6	BALB/c	NOD	129J
MHC haplotype	H-2 ^b	H-2 ^d	H-2K ^d D ^b	H-2 ^b
Receptor ¹²				
Ly-49A	✓	✓	✓	
Ly-49B	✓	✓	✓	
Ly-49C	✓	✓		
Ly-49D	✓		✓	
Ly-49E	✓		✓	✓
Ly-49F	✓			
Ly-49G	✓	✓	✓	✓
Ly-49H	✓			
Ly-49I	✓			
Ly-49J	✓			
Ly-49K	•			
Ly-49L	•	✓		
Ly-49M	•		✓	
Ly-49N	•			
Ly-49O				✓
Ly-49P			✓	✓
Ly-49Q	✓			
Ly-49R				✓
Ly-49S				✓
Ly-49T				✓
Ly-49U				✓
Ly-49V				✓
Ly-49W			✓	

Adapted from Kane, KP *et al.*, (2001) (74).

Table 1-2. NK cells from different strains have a different Ly-49 receptor expression profile. Some strains share alleles of the same Ly-49 receptors. Activating receptors are indicated in bold. In addition to the indicated Ly-49 receptors, Ly-49X has recently been cloned.



Adapted from Vivier E *et. al*, *Science* (2004) (136).

Figure 1-8 Signaling Pathways from NK receptors. NK signaling is a balance of positive signals from activating receptors, or negative signals from inhibitory receptors. Dotted arrows indicate inhibition or suppression, while full arrows indicate activation.

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Chapter 2 - Epitope mapping of Ly-49G and G-like receptors: CK-1 antibody defines a polymorphic site of functional interaction with class I ligand^{1,2}

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A. Introduction

Natural killer (NK) cells are large granular lymphocytes that constitute a major component of innate resistance to tumors or viruses (1, 2). Natural killer cells mediate their protective functions by direct cytotoxicity or release of cytokines and chemokines (3-5). Activities of NK cells are controlled through a balance between signals generated by inhibitory and activating receptors, with response resulting from reduction of inhibitory signals or enhancement of activating signals (6, 7).

Natural killer cells express inhibitory receptors that recognize class I MHC proteins, preventing NK cell aggression toward cells expressing normal levels of self class I MHC molecules (8, 9). Virally-infected cells and transformed cells typically express reduced levels of class I MHC products, rendering them susceptible to NK cell effector functions due to reduction or absence of class I dependent NK cell inhibitory signals. Natural killer cells express a variety of activating receptors that trigger NK cell-mediated cytotoxicity or cytokine release (7). Such receptors recognize ligands expressed on virally-infected, transformed or otherwise "stressed" cells (10).

In mice, NK cell receptors that directly recognize classical MHC I molecules are members of the *Ly-49* multigene family of lectin-like receptors (11). The *Ly-49* gene family is encoded within the NK gene complex on mouse chromosome 6 (12, 13). The number of *Ly-49* genes can vary in different inbred mouse strains, and *Ly-49* genes display extensive allelic variation (14). *Ly-49* receptors are disulphide-linked homo-dimers expressed on NK cells, NKT cells and some CD8⁺ memory T cells (15-17). The *Ly-49* family contains two receptor types, with inhibitory *Ly-49* receptors able to disrupt NK cell activation, while

activating Ly-49 receptors stimulate NK cell activation (11). Each subunit of an inhibitory Ly-49 receptor contains an immuno-receptor tyrosine-based inhibitory motif (ITIM)³ in its cytoplasmic tail. Following Ly-49 engagement with a class I MHC ligand, the ITIMs become phosphorylated, leading to SHP-1 tyrosine phosphatase recruitment and dephosphorylation of molecules in the NK cell activation cascade (18, 19). Ly-49 activating receptors generally share a high degree of amino acid identity in their ectodomains with inhibitory Ly-49 receptors, but lack an ITIM. Instead, activating Ly-49 receptors contain an arginine residue in the transmembrane segment that facilitates association with the signaling adapter protein DAP12 (20). Engagement of Ly-49 activating receptors stimulates NK cell signaling cascades associated with NK cell activation (21, 22).

In this report, we identified the amino acids controlling the specificity of two Ly-49G reactive antibodies, CK-1 and Cwy-3. We defined a critical region of functional interaction between the C57BL/6 allele product of the inhibitory Ly-49G receptor with a class I MHC ligand: the loop connecting the predicted $\beta 4$ and $\beta 5$ strands of Ly-49G. Our functional analyses employed native Ly-49 receptor ectodomains and ligands, and serological reagents to defined receptor epitopes. Thus, it was different from strategies that result in changes in primary amino acid sequences of the ectodomain, which could influence receptor conformation. In addition to distinguishing between Ly-49G allele products, we found that CK-1 recognized Ly-49M, but not the closely related Ly-49W receptor, possibly allowing the discrimination and characterization of NK cell subsets that express Ly-49M, but not one or more related activating Ly-49 receptors.

B. Materials and Methods

Antibodies

Hybridomas producing the following Abs: 4D11 (rat IgG2a), anti-Ly-49G (23); M1/42 (rat IgG2a), anti-mouse class I MHC (24); Y13-238 (rat IgG2a), anti p21^{ras} (25); and BB7.1 (IgG1), anti-HLA-B7 (26) were obtained from American Type Culture Collection (Manassas, VA). The Cwy-3 and CK-1 hybridomas were generated in this laboratory. The CK-1 (anti-Ly-49G, IgG1) hybridoma was generated following immunization of BALB/c mice with IL-2 activated C57BL/6 spleen cells, as described for the Cwy-3 (anti-Ly-49G, IgG1) hybridoma (27). Antibodies were prepared by ammonium sulfate precipitation, and PBS dialysis of tissue culture supernatants obtained from hybridomas grown in protein-free hybridoma medium. Purified OX-8 (IgG1) anti-rat CD8 α was purchased from BD PharMingen (San Diego, CA) (25). FITC-coupled mouse anti-rat and rat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified rat IgG was purchased from Sigma-Aldrich (Oakville, ON).

Cell lines

COS-7 SV40-transformed African green monkey kidney cells were grown in Opti-MEM I medium (Life Technologies, Burlington, ON), containing 10% heat-inactivated FCS (Invitrogen Canada, Burlington, ON) and 5×10^{-5} M 2-mercapto-ethanol. RNK-16, a spontaneous F344 rat strain NK cell leukemia (28) was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and 5×10^{-5} M 2-mercapto-ethanol (RNK medium).

Cloning of Ly-49G cDNA and site-directed mutagenesis

The cDNAs encoding the B6 and BALB/c alleles of Ly-49G, or Ly-49G-related receptors (Ly-49W1^{NOD}, Ly-49L^{BALB/c}, Ly-49M^{NOD}) were prepared by RT-PCR from total RNA isolated from IL-2-activated NK cells of corresponding strains, as described (29), then inserted into the *Xba*I/*Eco*RI sites of the mammalian

expression vector pCI-neo (Promega, Madison, WI). Ly-49G mutants were generated using the Stratagene QuikChange kit (Stratagene, La Jolla, CA).

Cell Transfection

The cDNA encoding inhibitory and activating Ly-49 receptors were transiently expressed in COS-7 cells using LipoFectamine (Life Technologies) as described (29). Vectors encoding activating receptors were co-transfected with a cDNA encoding mouse DAP12 in the pFLAG-CMV-1 expression vector (Sigma-Aldrich, St. Louis, MO). The generation of RNK-16 cells stably expressing Ly-49G2^{B6} (clone 1B6) or a chimeric receptor consisting of the ectodomain of Ly-49G2^{B6} fused to the transmembrane and intracellular domains of Ly-49W^{NOD} (Ly-49W/G^{B6}, clone 2G2) has been described previously (25).

Flow cytometric detection of Ly-49G and related receptors

Approximately 48 h after transfection to express Ly-49 receptors, COS-7 cells were incubated with 4D11, Cwy-3, CK-1 or isotype control antibodies M1/42 or BB7.1, respectively. The appropriate FITC-coupled secondary antibodies, mouse anti-rat or rat anti-mouse, were subsequently added for an additional incubation, whereupon samples were analyzed on a flow cytometer. To examine the expression of Ly-49G epitopes expressed on the surface of RNK-16 transfectants, Cwy-3, CK-1 or their isotype control BB7.1 were directly labeled with Alexa-Fluor® 488 or 647 fluorochromes (Molecular Probes, Eugene, OR), using the manufacturer's instructions. The RNK-Ly-49G^{B6} transfectant, 1B6, was resuspended at a density of 1×10^6 cells/ml and incubated with rat IgG at 40 µg/ml for 15 min to block rat Fc receptors. Saturating levels of Alexa-488 labeled Cwy-3 or BB7.1 antibodies were added to Fc receptor-blocked cells and incubated for 45 min, then saturating amounts of Alexa-647 labeled CK-1 or BB7.1 were added to the cells stained with the Alexa-488 labeled antibodies and incubated for 30 min. All incubations were conducted at 4°C. Cells incubated with labeled antibodies were fixed with 4 % *p*-formaldehyde in PBS, then analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA).

Cytotoxicity assays

Concanavalin A-activated blasts were prepared from DBA/2 and B6 strain spleen cells, as described (30). The Concanavalin A blasts were labeled at 37°C with 100–150 μ Ci of $\text{Na}^{51}\text{CrO}_4$ (^{51}Cr) (Mandel Scientific, Guelph, ON) for 1.5 h. Rat Fc receptors on RNK-16 Ly-49W/G^{B6} 2G2 effector cells were first blocked by incubation with rat IgG at 40 μ g/ml, then the effector cells were incubated for 30 min with medium, Ly-49G-specific antibodies, or isotype control antibodies prior to addition of labeled target cells. Target cells were incubated with 2G2 effector cells in the presence or absence of antibody for 4 h at 37°C in V-bottom microtiter plates at various E/T ratios in triplicate. After incubation, supernatant samples were counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer, Wellesley, MA). Percent specific lysis was determined as $(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100$. Cytotoxicity experiments were performed four separate times.

C. Results

Ly-49 receptor specificities of alloantibodies

Individual mouse NK cells can express several Ly-49 receptors simultaneously and this is determined by a stochastic process (31). Most described serological reagents that bind Ly-49 receptors are cross-reactive and recognize multiple Ly-49 receptors, making it difficult to attribute a ligand specificity or functional property to a particular Ly-49 receptor. Ly-49G and a number of Ly-49G-related activating receptors, Ly-49W, Ly-49M and Ly-49L are reactive with the 4D11 antibody. These different receptors share a high degree of amino acid identity within the carbohydrate recognition domain (CRD). Two allele products of *Ly-49G*, *Ly-49G2^{BALB/c}* and *Ly-49G2^{B6}*, share over 96% amino acid identity in their CRD. Nevertheless, strain-specific differences in *Ly-49* gene content and allelic variation offer opportunities to generate serological reagents with highly restricted *Ly-49* specificity. Such antibodies would be useful not only to detect receptor expression, but also to map receptor site(s) that are important for ligand interaction. We generated the antibody producing CK-1 hybridoma after injecting BALB/c mice with C57BL/6 IL-2 activated NK cells, similar to the generation of the Cwy-3 hybridoma (27). We compared the reactivity of CK-1, Cwy-3 and 4D11 with *Ly-49G* and *Ly-49G*-related receptors. COS-7 cells were transiently transfected with cDNAs encoding *Ly-49G* receptors from the C57BL/6 and BALB/c mouse strains, or *Ly-49G*-related activating receptors (*Ly-49L^{BALB/c}*, *M^{NOD}*, *W^{NOD}*) co-transfected with mouse DAP12. After 48 hours, the transfectants were stained with 4D11, Cwy-3, CK-1, or a corresponding isotype-matched control. All *Ly-49G* and *Ly-49G*-related receptors were strongly expressed on the COS-7 cells, as detected by substantial rat 4D11 antibody staining (**Fig. 2-1**, left panels). Since the Cwy-3 and CK-1 hybridomas were produced from the BALB/c mouse, it was expected that they would recognize the BALB/c allele product of *Ly-49G* poorly, if at all, as is the case (**Fig. 2-1**, middle, right panels). However, the Cwy-3 and CK-1 antibodies both recognized the *Ly-49G^{B6}* receptor (**Fig. 2-1**, middle, right panels), and CK-1 stained cell surface *Ly-49G2^{NOD}* (**Supp. Fig 2-1**). The Cwy-3 antibody also recognized the *Ly-49W*

activating receptor from the NOD strain as we previously reported (29), as well as another activator from the NOD strain, Ly-49M, but the BALB/c activating receptor Ly-49L only weakly, similar to the Ly-49G^{BALB/c} control (**Fig. 2-1**, middle panels). CK-1, like Cwy-3, recognized Ly-49M^{NOD} but, unlike Cwy-3, did not recognize Ly-49W^{NOD} or Ly-49L^{BALB/c} (**Fig. 2-1**, right panels). Thus, unlike 4D11 and Cwy-3, CK-1 has the ability to detect the expression of Ly-49M, as opposed to Ly-49W.

CK-1 and Cwy-3 epitope mapping

The differential reactivity of Cwy-3 and CK-1 for Ly-49G-related receptors suggested that they may bind distinct epitopes on these receptors. Residues contributing to strong Cwy-3 staining are not present in Ly-49G2^{BALB/c} or Ly-49L^{BALB/c}, but are present in Ly-49G2^{B6}, W and M. Similarly, the CK-1 epitope may involve residues in common to Ly-49G2^{B6} and Ly-49M^{NOD}, but not the other examined Ly-49G-related receptors. To identify candidate residues that could contribute to, or define the Cwy-3 or CK-1 epitopes, we examined stalk and CRD domains for amino acid residues that differed between the BALB/c and C57BL/6 alleles of Ly-49G. To further refine potential residues that contributed to the Cwy-3 and CK-1 epitopes, residues were considered that differed between the BALB/c and B6 alleles of Ly-49G and were also consistent with the patterns of Cwy-3 and CK-1 reactivity with Ly-49G-related receptors. There were four residues in the CRD at positions 159, 214, 251, and 267, which could contribute to the Cwy-3 or CK-1 epitopes (**Fig. 2-2**). Polymorphisms in the stalk regions did not fulfill the criteria for residues that may define Cwy-3 or CK-1 epitopes and the four identified residues were not in positions that could affect predicted receptor glycosylation sites.

We mutated the BALB/c allele of Ly-49G to contain each of the identified candidate residues in the B6 allele that may confer Cwy-3 or CK-1 antibody reactivities. The four single amino acid mutants were examined for reactivity by 4D11, Cwy-3 and CK-1. The 4D11 antibody recognized the wild type BALB/c and B6 allele products of Ly-49G, as well as all of the mutant BALB/c Ly-49G

receptors in transfected COS-7 cells (Fig. 2-3, left panels). This indicated that the mutant receptors folded in a manner similar to the wild type and expression of the mutant receptors was clearly detectable. More importantly, a single amino acid substitution at position 159 (D to N) of the BALB/c Ly-49G conferred recognition by the Cwy-3 antibody, but not by the CK-1 antibody (Fig. 2-3, middle and right panels). In contrast, a single amino substitution at position 251, (G to D) on a BALB/c template, resulted in CK-1 reactivity similar to that toward Ly-49G^{B6}, but not Cwy-3 reactivity (Fig. 2-3, middle and right panels). Substitutions of residues 214 and 267 did not result in Cwy-3 or CK-1 recognition (Fig. 2-3, middle, right panels), suggesting that residues at these positions do not contribute to the epitopes detected by Cwy-3 or CK-1. Thus, epitopes recognized by Cwy-3 and CK-1 can be conferred with single amino acid substitutions at positions 159 and 251, respectively. The epitopes recognized by Cwy-3 and CK-1 appear to be independent, since a change at position 159 only affected Cwy-3 recognition and a change at position 251 only affected CK-1 reactivity.

To confirm the importance of aspartic acid 251 for the CK-1 epitope, the C57BL/6 allele of the Ly-49G receptor was mutated at position 251 to glycine found at this position in the BALB/c allele of Ly-49G (D251G). This mutant receptor was expressed on the cell surface and folded properly, as demonstrated by 4D11 staining comparable to that of the B6 wild type receptor (Fig. 2-4, top and middle left panels). Substituting aspartic acid at this position with glycine results in a complete loss of CK-1 staining compared to the wild type Ly-49G^{B6} receptor (Fig. 2-4, right panels), but not Cwy-3 (Fig. 2-4 middle panels), demonstrating the critical role aspartic acid plays at this position for CK-1 recognition.

Ly-49G and Ly-49G-related receptors that are CK-1 positive such as Ly-49G^{B6} and Ly-49M contain the amino acid sequence DCD at positions 249-251, (Figs. 2-1 and 2-2). In addition, the mutant Ly-49G^{BALB/c} receptor (G251D) recognized by CK-1, also contains the 249-251 sequence, DCD. We directly tested a possible

requirement for simultaneous expression of aspartic acids at positions 249 and 251 to observe CK-1 reactivity. Thus, a second mutant was constructed of the Ly-49G2^{B6} allele, substituting an alanine for the aspartic acid at position 249. This mutant receptor was expressed on the cell surface similar to wild type Ly-49G2^{B6}, as detected by 4D11 staining (Fig. 2-4, bottom left panel), and Cwy-3 binding was not affected (bottom middle panel). However, the D249A mutant was not recognized by CK-1 (Fig. 2-4, bottom right panel). We conclude that recognition by CK-1 requires the presence of both aspartic acids at positions 249 and 251 in Ly-49G^{B6} and likely also Ly-49G related receptors.

The preceding experiments indicate that Cwy-3 and CK-1 antibodies have distinct requirements for the presence of specific amino acids in Ly-49 receptors for recognition. This raised the possibility that these antibodies recognize non-overlapping epitopes on Ly-49G^{B6}. To test this possibility directly, we determined whether these two antibodies could bind to Ly-49G^{B6} simultaneously. Ly-49G^{B6} transfected RNK-16 cells were stained with saturating concentrations of Cwy-3, CK-1, or both antibodies. The antibodies were labeled with distinct fluorochromes, Alexa 488 and Alexa 647. As expected, each Ly-49G^{B6} reactive antibody was able to bind the Ly-49G^{B6} transfectants (Fig. 2-5 lower left, upper right panels); in addition, both antibodies were able to stain the transfectants simultaneously (Fig. 2-5 lower right panel). No staining was observed with isotype controls (Fig. 2-5 upper left panel). Thus, Cwy-3 and CK-1 bind non-overlapping epitopes, consistent with the results of the mutagenesis experiments.

Differential inhibition of Ly-49G^{B6} ectodomain functional interaction with its ligand

We expressed a chimeric receptor which has the cytoplasmic and transmembrane domain of the Ly-49W activating receptor fused to the ectodomain of the inhibitory Ly-49G^{B6} receptor on the RNK-16 rat NK cell leukemia, as previously described (25). This approach provides a means to study Ly-49 inhibitory receptor interaction with its ligands, where recognition is detected as a direct positive NK cell response, instead of inhibition by the receptor of undefined

activating receptor functions (25). Significant expression of the chimeric Ly-49W/G^{B6} receptor on an RNK-16 transfectant clone 2G2, was demonstrated by staining with Cwy-3, CK-1 and 4D11 antibodies (**Fig. 2-6A**). No staining of RNK-16 cells with any of the three antibodies was detected (**Supp. Fig. 2-2**). Ly-49G^{B6} recognizes H-2D^d (25), and expression of the Ly-49W/G^{B6} chimera on RNK-16 cells results in cytotoxicity toward DBA/2 strain (H-2^d), but not C57BL/6 (H2^b) Con A blasts (25) (**Fig. 2-6B**, upper panel). We compared the ability of CK-1, Cwy-3 and 4D11 antibodies to block cytotoxicity mediated by the Ly-49W/G^{B6} chimeric receptor. As shown in **Figure 2-6B**, CK-1 and 4D11 were very effective at inhibiting recognition by the chimeric receptor bearing the Ly-49G^{B6} ectodomain, however, the Cwy-3 antibody was completely unable to interfere, even at high antibody concentrations (**Fig. 2-6B**, upper and lower panels). The inhibition of recognition by CK-1 was specific, since OX-8, an IgG1 isotype control that binds to CD8 α molecules on the surface of RNK-W/G^{B6} effector cells, did not affect recognition, whereas CK-1 significantly inhibited cytotoxicity (**Fig. 6B**, lower panel). Since the CK-1 and Cwy-3 antibodies bind the Ly-49G^{B6} ectodomain at independent sites, these data indicate that CK-1, but not Cwy-3 binds a site, like 4D11, that is important for ligand interaction. Moreover, since we have mapped the residues responsible for CK-1 binding to the polymorphic loop between the β 4 and β 5 strands, this indicates that this loop is involved in ligand recognition by Ly-49G^{B6}.

D. Discussion

Antibodies crossreactive for several Ly-49 family members and multiple alleles of a given Ly-49 gene have been generally useful in the study of Ly-49 receptor functions (23, 32). Since it has become apparent that the Ly-49 family contains twenty four or more members (14, 33), antibodies which identify a particular Ly-49 member or a narrow subset of receptors are needed to attribute expression or function to specific receptors. We generated antibodies that discriminate between Ly-49G alleles and Ly-49G-related Ly-49 family members, by taking advantage of known polymorphic differences between inbred mouse strains (14). We demonstrated that CK-1, similar to the Cwy-3 antibody, recognizes the C57BL/6, but not BALB/c allele product of Ly-49G. More importantly, CK-1 can discriminate Ly-49M from Ly-49W, activating receptors that are very similar to Ly-49G in their ectodomains. This level of discrimination is very high, since Ly-49W and Ly-49M share 96% amino acid identity. The Cwy-3 antibody and AT8 antibodies have been used together to characterize the functionality of NK cell subsets expressing specific Ly-49G allele(s) isolated from F₁ animals (35). The CK-1 antibody provides additional discrimination between Ly-49G-related receptors, and may be useful in discerning the specificities and functions of NK cell subsets in a broader range of mouse strains that express two Ly-49G alleles and/or Ly-49G related activating receptors. However, because CK-1 recognizes both Ly-49G^{NOD} (Supp. Fig. 2-1) and Ly-49M^{NOD}, it may be difficult to discriminate between these receptors on *ex vivo* NK cells from NOD mice. The significance of this limitation depends on the relative frequency of Ly-49G2 and Ly-49M expression on NOD strain NK cells, which remains to be determined.

We have identified the polymorphic amino acid residues of Ly-49G that confer binding specificity for the CK-1 and Cwy-3 antibodies. We modeled the location of the amino acids on Ly-49G in three dimensions, based on the known crystal structure of the related Ly-49A receptor (36). The CK-1 antibody requires the presence of two aspartic acids, D249 and D251, which reside on a loop between the β 4 and β 5 strand of Ly-49G^{B6}, and likely on other CK-1-recognized receptors

such as Ly-49M, for interaction (Fig. 2-7A). The $\beta 4$ - $\beta 5$ loop is highly polymorphic within the Ly-49 family with at least eleven different sequences identified. The Cwy-3 antibody requires an asparagine (e.g. Ly-49G^{B6}) or valine (e.g. Ly-49W), but not an aspartic acid (Ly-49G^{BALB/c}) at position 159. The N or V159 is located on the $\beta 1$ strand (Fig. 2-7A), a significant distance from the CK-1 epitope, but near the Ly-49 dimer interface, which is consistent with our observation that CK-1 and Cwy-3 can bind Ly-49G^{B6} simultaneously. Like Ly-49W1^{NOD}, Ly-49L^{BALB/c} contains a Val residue at position 159; yet, it is recognized very poorly if at all by Cwy-3. This may stem from the presence of a number of non-conservative amino acid substitutions within the Ly-49L receptor dimer interface in the CRD, which may alter the conformation of the receptor and, as a consequence, eliminate the Cwy-3 epitope or the accessibility of Cwy-3 for V159. The amino acids required for CK-1 or Cwy-3 recognition are predicted to be solvent exposed (Fig. 2-7A), thus they are unlikely to influence the folding or conformation of the receptor, nor are they in positions to alter the Ly-49 dimer interface. Rather, the required amino acids are likely to interact directly with the antibodies and contribute to the respective antibody combining sites. We did not map the common epitope recognized by the pan-specific 4D11 antibody, however, this epitope is not identical to those recognized by CK-1 or Cwy-3, since mutations that eliminated CK-1 or Cwy-3 binding did not affect binding by 4D11.

Mutagenesis and co-crystal structures have provided insight into how Ly-49A and C bind their high affinity class I ligands, H-2D^d and K^b, respectively (36-38). However, there have been no studies that define specific sites on Ly-49G that mediate class I recognition. Ly-49A and G are within the same Ly-49 subfamily (25, 39), and D^d is a shared ligand for both receptors. Thus, the co-crystal of Ly-49A bound to D^d may serve as an appropriate model for interaction of MHC I with Ly-49G or Ly-49G-related activating receptors. The Ly-49A-D^d co-crystal indicates two distinct areas of potential interaction, termed site 1 and site 2 and these sites are also suggested when modeling Ly-49G^{B6} binding to D^d (Fig. 2-7B).

Site 1 primarily involves a region on one end of the $\alpha 1$ and $\alpha 2$ domains of the MHC I molecule, whereas site 2 is a larger contact region beneath the peptide binding groove and includes a large cleft formed by portions of the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains of the MHC I heavy chain, and $\beta 2m$. The Site 2 region has been shown to be important for Ly-49A and C interactions with class I ligands; this may also be the case for Ly-49G. Focusing on site 2, co-crystal structures of Ly-49A and C with their respective ligands indicate that Ly-49A and C share a group of conserved residues within the Ly-49 family, that include S236, R239 and D/E241, which form a patch that interacts with class I ligands. Ly-49A has multiple additional contacts with class I and $\beta 2m$ at site 2, beyond those observed with Ly-49C, particularly with residue Q29 of $\beta 2m$ (36). These additional contacts are largely mediated by variable residues within the Ly-49 family, particularly of the $\beta 4$ - $\beta 5$ loop (40). The epitope recognized by Cwy-3 maps to a site on Ly-49G^{B6} that is distant from potential sites of class I interaction (**Fig. 2-7B**), and is consistent with our observation that this antibody is unable to block a functional interaction of Ly-49G^{B6} with D^d. It should be mentioned that Cwy-3 can block Ly-49G^{B6} interaction with a class I ligand, but only when a large multivalent molecule such as protein G is used as a secondary reagent, presumably enhancing steric hindrance (25). In contrast, CK-1 binds residues of the Ly-49G^{B6} $\beta 4$ - $\beta 5$ loop, which are predicted to directly interact with class I at site 2, and site 1 (**Fig. 2-7B**), and we have shown that CK-1 is quite effective at disrupting functional MHC I interaction with the Ly-49G^{B6} ectodomain. Since the $\beta 4$ - $\beta 5$ loop is distinct from the conserved 236-241 patch, and utilized by Ly-49A but not Ly-49C for MHC I interaction, our results with CK-1 suggest that Ly-49G binds D^d in a manner similar to Ly-49A, but not Ly-49C. We have also shown that the $\beta 4$ - $\beta 5$ loop is important in determining the class I allele specificity of Ly-49G-related activating receptors by mutagenesis (40). The effectiveness of CK-1 in blocking responses and the mapping of its epitope to the $\beta 4$ - $\beta 5$ loop is complementary to results obtained with Ly-49 mutagenesis and co-crystal structures (36-38), and

emphasizes the importance of the $\beta 4$ - $\beta 5$ loop in MHC I recognition by Ly-49A and G receptors.

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E. Individual Contributions: M. Osman and K. Kane wrote and modified the paper for publication. The initial predictions regarding which residues to mutate were made by Dr. E. Silver; these were corroborated and updated by M. Osman in conjunction with Dr. K. Kane. Experimentally, Dr. E. Silver generated the wild type cDNA constructs used by J. Varghese to generate the mutant Ly-49G receptors via site-directed mutagenesis. M. Osman expressed the mutant Ly-49 receptors in COS-7 cells, assessed the relative binding affinities of the anti-Ly49G antibodies to the mutant receptors, and the antibody competition experiments using flow cytometry. He also assisted with the modeling of the Ly-49G structure crystal in collaboration with Dr. G. Audette in Dr. B. Hazes's lab. Dr. E. Silver also generated the stable RNK-16 stable transfectants. Dr. C. Chang generated both the Cwy-3 and CK-1 monoclonal antibodies, and D. Gong performed chromium release cytotoxicity assays.

³ **Abbreviations:** β 2m, β 2-microglobulin; ^{51}Cr , $\text{Na}^{51}\text{CrO}_4$; CRD, carbohydrate recognition domain; E/T ratio, effector to target ratio; ITIM, immuno-receptor tyrosine-based inhibitory motif; PDB, protein database.

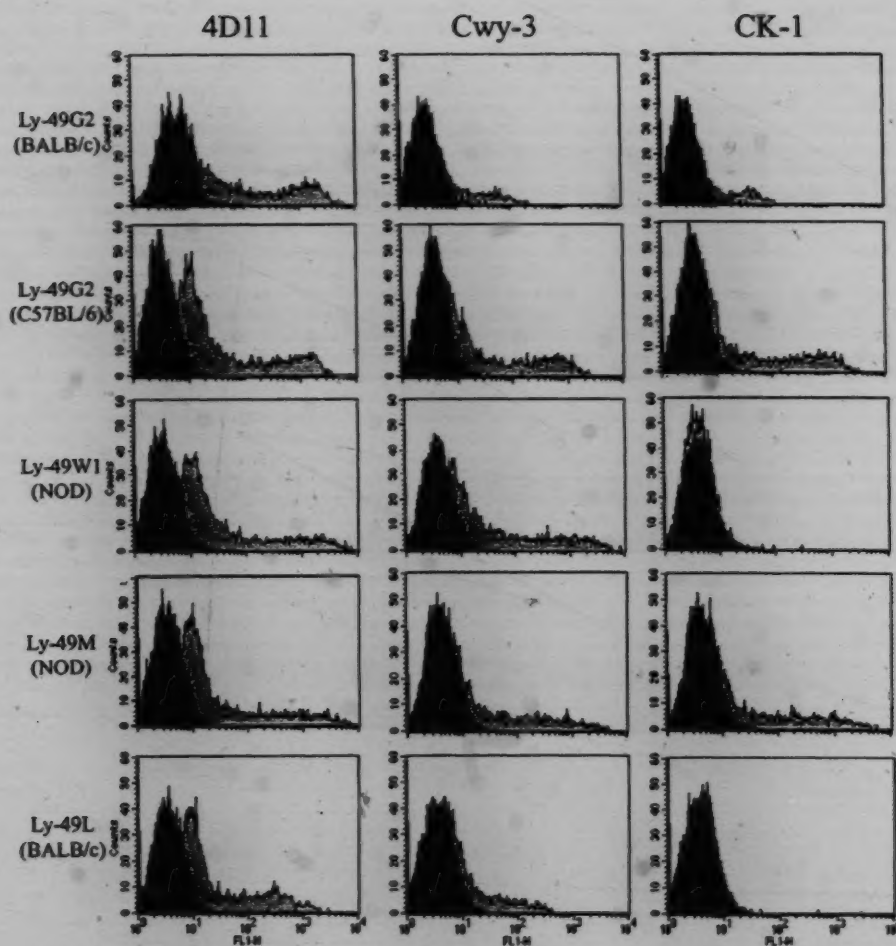


Figure 2-1 The CK-1 and Cwy-3 antibodies recognize partially overlapping sets of Ly-49 receptors. COS-7 cells were transiently transfected to express the inhibitory Ly-49G2 receptor from C57BL/6 or BALB/c mice, or Ly-49W1, Ly-49M, or Ly-49L receptors and murine DAP12, then analyzed by flow cytometry using 4D11, Cwy-3, or CK-1 antibodies (gray), or isotype controls, M1/42 and BB7.1 (black).

Function	Receptor	Strain	← cytoplasmic → ← -TM	
I	Ly-49G2	(BALB/c)	MSEQEVTYSTVRFHSSRLQKLVRTEEPQRPREACYRKYSVPWKLVIACG	51
I	Ly-49G2	(C57BL/6)	-----E-----	
A	Ly-49W1	(NOD)	-----F-A-----K-G-NR-L-TGK-QK-GL-VC-----Q-----L-----	
A	Ly-49M	(NOD)	-----F-A-----K-G-NR-L-TGK-K-GL-***-----Q-----L-----	
A	Ly-49L	(BALB/c)	-----F-A-----K-G-NR-L-TGK-K-GL-***-----Q-----L-----	
			----- stalk -----	
I	Ly-49G2	(BALB/c)	IFCPLLLVTVALLAITIFQHIQKKHELQETLNCHDNCSTT*QSDVNLKDEL	101
I	Ly-49G2	(C57BL/6)	-----L-----*-----S-----P-----	
A	Ly-49W1	(NOD)	-----LIS-R-----I-SV-*VN-----NS-M-----K-----T-----I-----	
A	Ly-49M	(NOD)	-----LIS-R-----I-SV-*VN-----NS-----K-----T-----I-----	
A	Ly-49L	(BALB/c)	-----LIS-R-----I-SV-*VN-----NS-N-----K-----T-----I-----	
			----- stalk -----	
I	Ly-49G2	(BALB/c)	LRNKSIECRPGNDLLESINRDQKRWYSETKTFSDSSQHTGRGFEKYWFCYG	153
I	Ly-49G2	(C57BL/6)	-----S-----N-----	
A	Ly-49W1	(NOD)	-----SST-----HKE-N-----	
A	Ly-49M	(NOD)	-----T-----KE-N-----R-----T-----	
A	Ly-49L	(BALB/c)	-----SST-----HKE-N-----	
			----- CRD -----	
I	Ly-49G2	(BALB/c)	IKCYIFDMDRKTWSGCKQTCQISSLSLLKIDNEDELKFLQNLAPSDISWIG	204
I	Ly-49G2	(C57BL/6)	-----H-----	
A	Ly-49W1	(NOD)	-----V-----	
A	Ly-49M	(NOD)	T-----V-----D-----	
A	Ly-49L	(BALB/c)	-----V-----KL-V-----SC-----	
			----- CRD -----	
I	Ly-49G2	(BALB/c)	FSYDNKKKDWANIDNGPSKLALNTTKYNIRDGLCMSLSKTRLDNGDCGKSY	255
I	Ly-49G2	(C57BL/6)	L-----V-----D-----	
A	Ly-49W1	(NOD)	-----V-----	
A	Ly-49M	(NOD)	-----V-----D-----	
A	Ly-49L	(BALB/c)	L-----N-----M-----G-----L-----DN-D-----F-----	
			----- CRD -----	
I	Ly-49G2	(BALB/c)	ICICGKRLDKFPT	267
I	Ly-49G2	(C57BL/6)	-----H-----	
A	Ly-49W1	(NOD)	-----H-----	
A	Ly-49M	(NOD)	-----S-----H-----	
A	Ly-49L	(BALB/c)	-----H-----	

Figure 2-2 Amino acid alignment of Ly-49G and Ly-49G-related receptors from different mouse strains. Regions corresponding to the cytoplasmic, transmembrane (TM), stalk and CRD are indicated with arrows. Amino acids in bold identify residues that differ between BALB/c and B6 allele products of Ly-49G2 and are consistent with the staining patterns of Ly-49G and related receptors. Determined or expected receptor function is designated by (I) inhibitory, or (A) activating. GenBank accession numbers: AF307946 (Ly-49G2^{BALB/c}), NM 014194 (Ly-49G2^{C57BL/6}), AF283250 (Ly-49W1^{NOD}), AF283252 (Ly-49M^{NOD}) and AF307947 (Ly-49L^{BALB/c}).

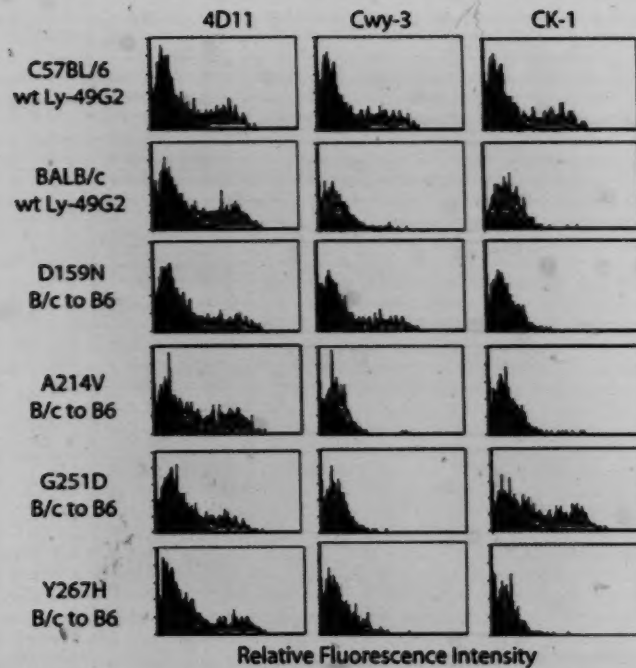


Figure 2-3 Recognition by Cwy-3 and CK-1 antibodies requires distinct Ly-49G^{B6} amino acid residues. COS-7 cells were transfected with Ly-49G2 from BALB/c or C57BL/6 mice, or mutants that substitute individual Ly-49G^{B6} residues for Ly-49G^{BALB/c} residues, then analyzed by flow cytometry using 4D11 to monitor Ly-49G surface expression (gray) or the M1/42 isotype control (black). Alternatively, transfected cells were analyzed using Cwy-3 or CK-1 (gray), or an isotype control (BB7.1, black), to identify mutants that conferred the epitope recognized by either Ly-49G^{B6} binding antibody.

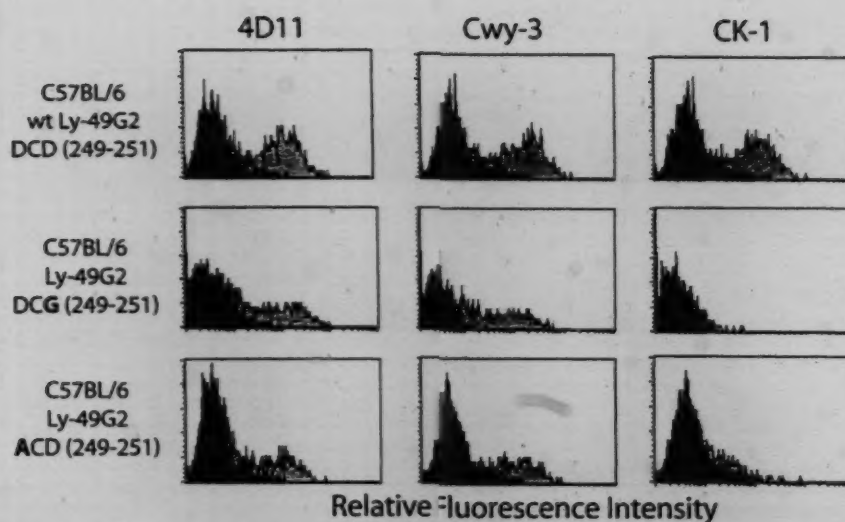


Figure 2-4 CK-1 recognition requires two aspartic acid residues, 249 and 251 of Ly-49G2^{B6}. COS-7 cells were transfected with wild type Ly-49G2^{B6} or amino acid substitution mutants of Ly-49G2^{B6} as indicated, then analyzed by flow cytometry using 4D11 (gray) or an M142 isotype control (black), to monitor Ly-49G^{B6} expression. Cwy-3 or CK-1 (gray), or an isotype control (BB7.1, black) staining was performed to identify Ly-49G^{B6} mutants that affected the CK-1 recognized epitope. Differences in $\beta_4\beta_5$ loop amino acid sequences of mutant receptors are in bold.

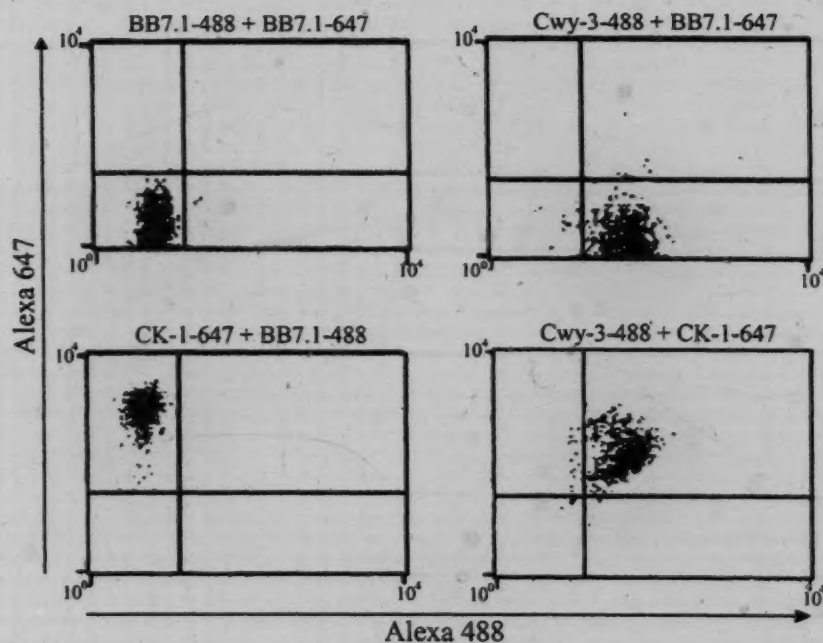


Figure 2-5 CK-1 and Cwy-3 recognize distinct non-overlapping epitopes. RNK-16 cells stably transfected with Ly-49G2^{B6}, clone 1B6, were pre-incubated with rat IgG to block antibody binding to cell Fc receptors. 1B6 cells were treated with Alexa-488 labeled antibodies (Cwy-3, or BB7.1) followed by treatment with Alexa-647 labeled antibodies (CK-1 or BB7.1), as indicated. Alexa-488 staining is indicated on the horizontal axis, while Alexa-647 staining is on the vertical axis. All antibodies were added at saturating concentrations.

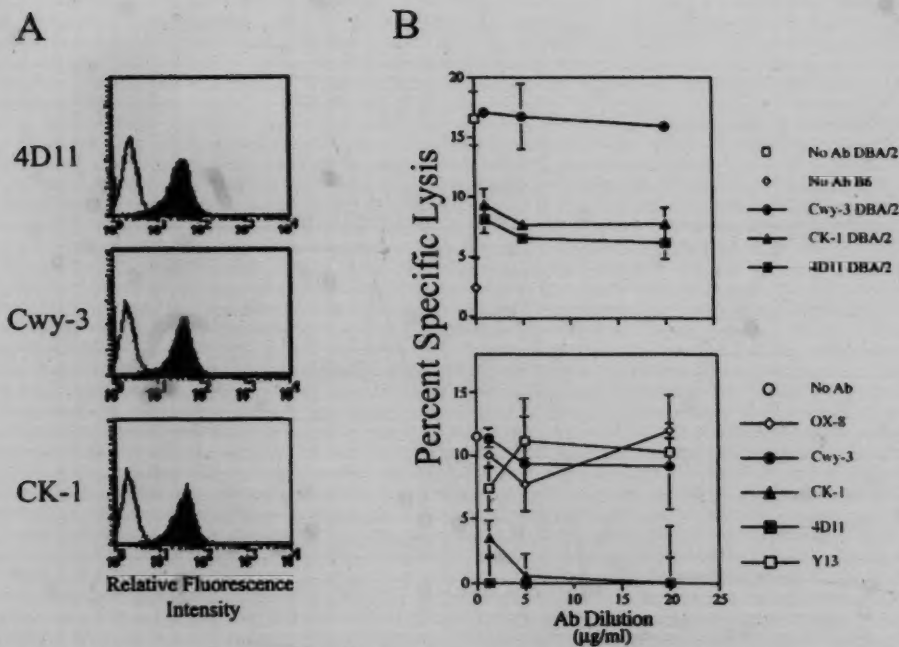


Figure 2-6 Epitope dependence of Ly-49G interaction with ligand. A, Expression of the Ly-49W/G^{B6} chimera on RNK-16 transfectant 2G2, detected with Cwy-3, CK-1 and 4D11 antibodies (filled), isotype controls BB7, Y13 respectively (unfilled); B, Effect of Cwy-3, CK-1 and 4D11 antibodies on the inhibition of Ly-49G2^{B6} recognition of target cells bearing its MHC I ligand. Lysis of DBA/2 Con A blasts by RNK.WG^{B6} clone 2G2, was measured with no Ab or in the presence of anti-Ly-49G Ab (Cwy-3, CK-1 or 4D11, upper and lower panels) or control Abs (OX-8 or Y13-238, respectively, lower panel), at the indicated concentrations. C57BL/6 Con A blasts were negative control target cells (upper panel). The FcR of effector cells were blocked by incubation with rat IgG (40 $\mu\text{g/ml}$) prior to addition of the indicated antibodies. Lysis was determined in standard 4-hr release assay using an E:T ratio of 12.5:1. Antibodies were at the indicated concentrations during the assay. Data represent the mean of triplicate wells, with error bars representing the respective standard deviations.

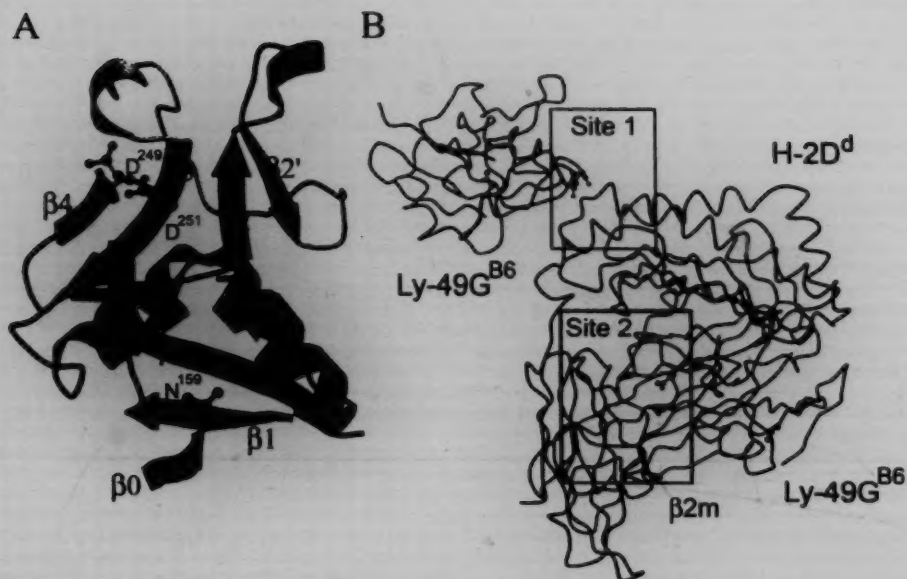
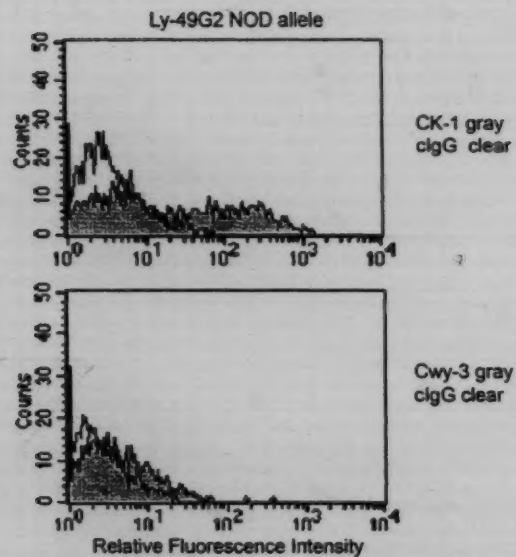
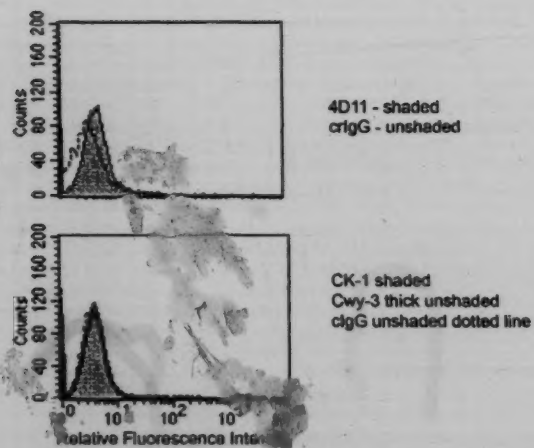


Figure 2-7 Location of Ly-49G residues that determine CK-1 and Cwy-3 binding and their interaction with H-2D^d. A, Model of the Ly-49G^{B6} monomer, based on the Ly-49A crystal structure (PDB ID # 1QO3; (36)). Residues D249, D251 (CK-1 epitope) and N159 (Cwy-3 epitope) are shown in ball-and-stick form with yellow and blue bonds, respectively. B, Interaction of Ly-49G^{B6} with D^d, based on Ly-49A-D^d complex (PDB ID # 1QO3, (36)). H-2D^d heavy chain, $\beta 2$ -microglobulin, and the Ly-49G^{B6} monomers are shown in blue, green and red, respectively, and the interactions at sites 1 and 2 are boxed. Ly-49G^{B6} residues determining CK-1 (yellow) and Cwy-3 (blue) specificity are shown in ball-and-stick form. Images were created using the programs MOLSCRIPT (41), and Raster3d (42).



Supplementary Fig. 2-1. Ly-49G2^{NOD} is positive for staining with CK-1, but not Cwy-3. COS-7 cells were transiently transfected with 2 μ g of Ly-49G2^{NOD}-pCI-neo as described in the *Materials and Methods* section, then receptor surface expression was assessed using flow cytometry using either CK-1, Cwy-3 or BB7.1 as an isotype-matched control antibody.



Supplementary Figure 2-2. RNK-16 cells are not recognized by 4D11, CK-1 or Cwy-3. RNK-16 cells were stained with 4D11, Cwy-3, CK-1 or corresponding isotype-matched control as described in the *Materials and Methods* section, then analyzed using a FACScan flow cytometer.

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Chapter 3 - Activating Ly-49 receptors regulate LFA-1 mediated adhesion by NK cells^{1,2}

Mohammed S. Osman, Deborah N. Burshtyn and Kevin P. Kane

A. Introduction

Natural killer (NK) cells are large granular lymphocytes that are important components of innate resistance to tumors and viruses (1, 2). Natural killer cells mediate protective functions through cytolysis of virally infected cells or tumor cells, and by release of cytokines and chemokines (3-5). NK cells are controlled through a balance of signals generated by inhibitory and activating receptors, with response resulting from reduction of inhibitory signals or enhancement of activating signals (6).

NK cells express a variety of activating receptors that trigger cell-mediated cytotoxicity and cytokine release (6). Activating NK cell receptors of mouse that trigger cell-mediated cytotoxicity and/or cytokine release include FcγRIII, natural cytotoxicity receptors, NKG2D, and activating Ly-49 receptors that directly recognize MHC-I molecules (6-12). Activating Ly-49 molecules are disulphide-linked homodimeric lectin-like receptors that contain an arginine residue in the transmembrane segment which facilitates association with the signaling adapter protein DAP12 (13). DAP12 contains an ITAM, which recruits Syk family tyrosine kinases to trigger the cytolytic cascade and cytokine release (14).

NK cells also express LFA-1 ($\alpha_L\beta_2$), a β_2 -integrin that binds ICAM-1-5 and is important for adhesion to target cells (15-17). In various cell types, signals emanating from activating receptors regulate the affinity and/or avidity of LFA-1 ultimately resulting in increased binding of ICAM-1 (18-20). LFA-1 binding of ICAM-1 stabilizes the intercellular adhesion between cytotoxic cells and their targets promoting the delivery of cytotoxic granules contents toward susceptible targets (15, 21). Inside-out signaling producing firm LFA-1 mediated adhesion, for example, between cytotoxic T cells and their targets, is triggered by signaling resulting from TCR engagement (19). In NK cells, LFA-1 provides adhesion and

contributes an early activating signal that facilitates polarization of cytolytic granules toward target cells (15, 17, 21). While the signaling by activating Ly-49 engagement has some similarities with that by the TCR, such as involvement of an ITAM and Syk family kinases, there are no reports to date demonstrating inside-out signaling by an NK cell activating receptor.

In this study we show that Ly-49 activating receptors promote adhesion of NK cells through a DAP12-dependent inside-out regulation of LFA-1 binding to ICAM-1. Furthermore, we find that cross-linking with Ly-49 receptor-specific antibodies can mimic cognate ligand interaction and trigger LFA-1 mediated NK cell binding to purified ICAM-1. Together, these results establish a role for Ly-49 activating receptors in regulating adhesion to target cells through LFA-1.

B. Materials and Methods

Antibodies and cell lines

The following antibodies were produced from the hybridomas as described (22) A1 (IgG2a), anti-Ly-49A/P (23); B27M1 (IgG2a), anti-HLA-B7 (24); M1/42, anti-mouse H-2 (rat IgG2a) (25); 2.4G2 (rat IgG2b) anti-mouse Fc- γ receptor (26); M17/5.2 (rat IgG2b) anti-mouse LFA-1 (27); and anti-mouse F4/80 antigen (rat IgG2b) (28). Purified G28, (IgG2a) anti-rat CD8 α ; WT.1, (IgG2a) anti-rat CD11a; and 4E5, (rat IgG2b), anti-Ly-49D-FITC were purchased from BD/PharMingen (San Diego, CA). FITC-coupled rat anti-mouse IgG, mouse anti-rat IgG and APC coupled polyclonal anti-human IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified γ m³, γ ar, and unmodified rat IgG were purchased from Sigma-Aldrich (Oakville, ON). Rabbit anti-DAP12 serum (14), was provided by Dr. D. McVicar (Frederick, MD, USA). The pharmacological inhibitors PP2 and piceatannol were purchased from Calbiochem (San Diego, CA), and reconstituted in DMSO then stored at 4°C. RNK-16, a spontaneous rat NK leukemia was maintained as described (22, 29). The P815 mouse mastocytoma was maintained in RPMI 1640 supplemented with 5% FCS and 5×10^{-5} M 2-ME.

Flow Cytometry

For the detection of cell surface Ly-49P, CD8 α , or LFA-1, RNK or RNK transfectants ($\sim 0.3 \times 10^6$ cells/sample) were incubated with normal rat serum for 10 min at RT, then the indicated mAb was added (2 μ g/mL) for 30-45 min on ice. Following the incubation, the cells were washed three times using 1XPBS, then FITC-coupled secondary rat anti-mouse IgG antibodies were added, incubated for 20-30 min on ice, washed three time with 1XPBS, fixed with 4% *p*-formaldehyde in PBS then analyzed using a FACScan® flow cytometer. For the detection of Ly-49D on *ex vivo* NK cells, 4E5-FITC (2 μ g/mL) was added to DX5-enriched NK cells pre-incubated with 2.4G2 (10 min RT, 10 μ g/mL) for 30 min on ice, washed three times with 1XPBS, then fixed and analyzed as above.

Mutagenesis of Ly-49P

Ly-49P (22), was mutated using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) to encode Leu for Arg 57 in *Ly-49P*. The altered cDNA was verified by DNA sequencing and inserted into *XhoI/XbaI* sites of the BSR α EN vector (provided by Dr. A. Shaw [Washington University, St. Louis, MO]). RNK-16 were transfected with wild type or mutant *Ly-49P* cDNAs as described (22), to generate RNK-P and RNK-P(R57L).

Immuno-blotting

$1.5\text{--}2.0 \times 10^7$ cells were lysed in 1% Triton w/v, 0.15 M NaCl, 20 mM Tris pH 8 with protease inhibitors. Lysates were immunoprecipitated with A1 or B27M1 and protein G-agarose, separated by 12% SDS-PAGE under reducing conditions, and transferred to Immobilon-P. Membranes were blotted with rabbit antiserum and detected by chemiluminescence (Pierce; Rockford, IL).

Cytotoxicity assays

Target cells were labeled at 37°C with 100 μCi of $\text{Na}^{51}\text{CrO}_4$ for 1-2 h. Following washing, 1×10^4 ^{51}Cr -labeled targets were incubated for 4 h at 37°C in V-bottom microtiter plates with RNK-16, RNK-P, or *Ly49P*(R57L) at various E:T ratios in triplicate. Following the incubation, supernatant samples were counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer, Wellesley, MA). Percent specific lysis was determined as (experimental release - spontaneous release)/(maximum release - spontaneous release) $\times$ 100. Cytotoxicity experiments were performed at least separate times.

Animals

Female C57BL/6 mice at 6-8 wk of age were purchased from the Jackson Laboratory (Bar Harbor, ME). Experiments were approved by the Animal Welfare and Policy Committee of the University of Alberta (Edmonton, Alberta, Canada).

Conjugate assays

The cell conjugate assays were performed as described (20), using red (PKH 26) and green (PKH67) membrane linker dyes (Sigma-Aldrich; Oakville, ON). For assays in the presence of blocking mAbs, the antibodies were pre-bound to soluble protein A/G (1:1 ratio) for 15 minutes at room temperature (RT), then added to RNK-16, RNK-P or RNK-P(R57L) effector cells and incubated at 4°C for 10 min. Following incubation, labeled target cells were added to the effector cells pre-incubated with normal rat serum, then the number of tight conjugates was determined (30). Results represent the means $\pm$ SD. For adhesion assays with *ex vivo* NK cells, mouse ICAM-1 was affinity-purified from mouse A20 cells using a YN-1/1.7.4 sepharose column as described (31). NK cells were prepared using the Easy Sep DX5⁺ selection kit (Stem Cell Technologies; Vancouver, BC) from RBC-depleted single cell suspensions of C57BL/6 spleens. Cells were stained for Ly-49D expression using 4E5-FITC antibodies. DX5⁺ cells were treated with α -Ly-49D-FITC or α -H2 mAbs (1 μ g/10⁶ cells), then mixed with cell-size beads immobilized with BSA or ICAM-1 (2 μ g/10⁷ beads) in RPMI containing 0.5 mM MgCl₂ at an effector:bead ratio of 1:1, and centrifuged at 30xg for 3 min. Cell/bead mixtures treated with mAbs were cross-linked using gar (0.5 μ g/mL) for 2.5 min, then fixed with 1% *p*-formaldehyde. Ly-49D⁺ cell conjugates were determined as the percentage of DX5⁺/Ly49D⁺ cells shifted to a SSC corresponding to that of the beads, as illustrated in Fig. 5A and depicted in Fig. 5C.

Plate ICAM-1 adhesion assay

Purified ICAM-1 was immobilized on flat-bottom microtitre plates at 1 μ g/mL overnight at 4°C in PBS supplemented with 900 μ M CaCl₂ and 500 μ M MgCl₂. RNK-16, RNK-P or RNK-P(R57L) cells were labeled with ⁵¹Cr, incubated with normal rat serum for 10 min at RT, then with 5 μ g/mL of A1 or isotype control antibodies (as indicated) for 10 min at RT, and the cells (1.1 $\times$ 10⁵) were added to immobilized ICAM-1 or 2% FCS, with or without 0.5 μ g/mL gam for 30 min at 37°C. At the end of incubation, an aliquot was harvested to determine

spontaneous release, then unbound cells were removed by pipetting shear force, and the number of bound cells determined by β -counting. In all adhesion experiments, cell binding was calculated as: percent specific cell binding = $100 \times [\text{bound counts} / (\text{total counts} - \text{spontaneous release})]$, with results representing the mean $\pm$ SD of each triplicate. For experiments using pharmacological inhibitors, ^{51}Cr -labeled RNK-P cells were pre-treated with the indicated concentration of the inhibitor or DMSO alone for 30 min at 37°C . Following incubation with either inhibitor, the cells were treated as above with normal rat serum and A1, then added to immobilized ICAM-1-immobilized wells containing γm for 30 min at 37°C . Subsequently, the cells were harvested as above, and the number of bound cells was determined by β -counting.

C. Results

Ly-49P dependent lysis requires LFA-1

To study the potential role of activating Ly-49 receptors in triggering NK cell adhesion to target cells, we expressed the non-obese diabetic (NOD) activating Ly-49P on rat RNK-16 leukemia cells by transfection and ensured that the transfectants expressed similar levels of CD11a and CD8 α as parental cells (Fig 3-1A, upper panels). To monitor the expression of Ly-49P on the surface of RNK-16 cells, we used the A1 mAb which we have previously shown to recognize both Ly-49A and Ly-49P (22). RNK cells transfected with Ly-49P (RNK-P) expressed uniform levels of Ly-49P as expected, while no mouse Ly-49P expression was detected on the untransfected rat cells (Fig 3-1A, lower panels). We employed P815 mastocytoma cells as target cells for the Ly-49P transfectants, since they express endogenous H-2D^d and ICAM-1, ligands for Ly-49P and LFA-1, respectively (Fig. 3-1A, far right panel). The P815 target cells were efficiently lysed by RNK-P effector cells, but not parental RNK-16 (Fig. 3-1B). These results indicate that lysis of target cells is dependent on Ly-49P recognition of H-2D^d expressed by P815. Lysis of P815 was blocked by antibodies to Ly-49P, as well as LFA-1 (Fig. 3-1C) and ICAM-1 (Fig. 3-1D), but not by isotype control antibodies (Fig. 3-1C and 3-1D), indicating that by interacting with their respective ligands, Ly-49P and LFA-1 both contribute to P815 lysis.

Ly-49P promotes adhesion to susceptible target cells in an LFA-1 dependent manner

Studies using cytotoxic T cells have demonstrated that tight adhesion is an important step preceding cytotoxicity (15). Therefore, we tested whether Ly-49P promotes formation of conjugates between effector cells and susceptible targets, using two color flow cytometry (30). RNK-P, but not parental RNK-16, rapidly formed tight conjugates with P815, with maximum hetero-conjugates detected by ten minutes (Fig. 3-2A). Cell adhesion was dependent on engagement of Ly-49P

with H-2D^d, as blocking this interaction significantly reduced the number of tight conjugates (**Fig. 3-2B**). The tight cell adhesion was also dependent on interactions between LFA-1 and ICAM-1 as antibodies to these molecules also reduced the number of NK cell conjugates (**Fig. 3-2B and 3-2C**). Thus, Ly-49P-mediated recognition regulates cell binding in a LFA-1 – ICAM-1 dependent manner, and promotes cytotoxicity of susceptible target cells.

Ly-49P-mediated adhesion is DAP12 dependent

The interaction between inhibitory Ly-49 and MHC-I is sufficient to mediate binding of cells to ligands on plates or on other cells (32, 33). To distinguish the contribution of direct binding of H-2D^d by Ly-49P toward tight cell:cell adhesion, from Ly-49P-mediated signaling events that alter LFA-1 binding, we constructed an Ly-49P mutant with an amino acid substitution at position 57, where the positively charged Arg was replaced with Leu and stably expressed this receptor on RNK-16 cells by transfection. This mutation, R57L, abrogates the ability of the receptor to associate with DAP12, and in turn its ability to signal following ligand recognition, but still allow Ly-49P(R57L) surface expression (13). RNK-P(R57L) cells expressed similar levels of Ly-49P using the A1 mAb, as well as CD11a, and CD8 α as RNK-P cells (**Fig 3-3A**), and **Supp. Fig. 3-1**). However, the DAP12 signaling adapter co-immunoprecipitates with Ly49P but not Ly-49P(R57L) from the RNK transfectants (**Fig. 3-3B**). Furthermore, RNK-P(R57L), unlike RNK-P, was unable to lyse P815 (**Fig. 3-3C**), despite retaining the ability to lyse YB2/0 directly and EL-4 cells by ADCC via CD16 (**Supp. Fig. 3-1**). Importantly, the tight adhesion typically observed between RNK-P and P815 was instead negligible between RNK-P(R57L) and P815 and similar to background levels obtained with untransfected RNK-16 (**Fig. 3-3D**). These results indicate that the ligand binding capacity of Ly-49P does not significantly increase adhesion to H-2D^d bearing target cells on its own, and instead suggests that Ly-49P promotes tight binding to susceptible target cells through DAP12-dependent signals.

Cross-linking of Ly-49P leads to DAP12 dependent inside-out signals regulating LFA-1 binding to mouse ICAM-1

To directly test whether Ly-49P could trigger changes in LFA-1 binding to ICAM-1 we examined binding of RNK-P to plate bound mouse ICAM-1, upon stimulation of Ly-49P. To mimic signals generated by Ly-49P recognition of H-2D^d, we cross-linked Ly-49P, Ly-49P(R57L) or CD8 α expressed on RNK-16 or its transfectants with primary antibodies specific for Ly-49P or rat CD8 α , with secondary gam antibody, then determined cell binding to mouse ICAM-1. Little binding was evident to wells immobilized with serum, or following CD8 α cross-linking (**Fig. 3-4A**). RNK-P, but not untransfected RNK-16 or RNK-P(R57L), bound to immobilized ICAM-1 when stimulated by anti-Ly-49P (A1) and secondary antibodies (**Fig. 3-4A**). Furthermore, crosslinking with secondary antibodies was required (**Supp. Fig. 3-2**) – suggesting that activating Ly-49 clustering may promote events leading to induced ICAM-1 binding. RNK transfectants stably expressing the activating Ly-49D receptor also bound immobilized ICAM-1 following cross-linking with anti-Ly-49D and secondary antibodies (**Supp. Fig. 3-2**). Therefore, engagement of activating Ly-49 receptors on RNK-16 cells triggers DAP12-dependent LFA-1 binding to ICAM-1. Furthermore, RNK-P triggered binding to ICAM-1 required both Syk and Src family kinase (SFK) activities as the specific inhibitors piceatannol and PP2, respectively, significantly inhibited binding of RNK-P cells to ICAM-1 in a dose-dependent manner (**Fig. 3-4B**).

Ex vivo NK cells regulate LFA-1 binding to ICAM-1 via Ly-49D

To determine whether triggering of LFA-1 binding to ICAM-1 is a property of an activating Ly-49 receptor expressed by normal mouse NK cells, we developed a flow cytometry based ICAM-1 binding assay using resting *ex vivo* NK cells. NK cells enriched from C57BL/6 spleen by DX5 mAb selection, of which 25-30% express the activating Ly-49D receptor (34), were stimulated with α -Ly-49D or an α -H-2 control and secondary antibody in the presence of ICAM-1 or BSA beads. The number of DX5⁺/Ly-49D⁺ NK cells of each experimental group, bound to the beads immobilized with ICAM-1 or BSA was determined, as

diagrammed (**Fig. 3-5A**). The ICAM-1 was efficiently displayed on the cell size beads as detected by flow cytometry with the YN1/1.7.4 antibody (**Fig. 3-5B**), and beads were easily distinguished from NK cells due to very different forward and side scatter properties (**Fig. 3-5B**). Conjugates between beads and DX5⁺/Ly49D⁺ NK cells were readily detected by a shift to enhanced side scatter of gated DX5⁺/Ly-49D⁺ cells, as a consequence of bead binding (**Fig. 3-5C**). Whereas only 4% of Ly-49D⁺ NK cells stimulated with the control α -H2 antibody bound ICAM-1 beads, approximately 23% of Ly-49D⁺ NK cells bound ICAM-1 beads upon stimulation by Ly-49D engagement (**Fig. 3-5C and 3-5D**). These results show that the specific engagement of an activating Ly-49 NK cell receptor expressed on *ex vivo* NK cells up-regulates NK cell adhesion to ICAM-1. Furthermore, the adhesion to ICAM-1 by the *ex vivo* NK cells was mediated by LFA-1 as antibodies to this receptor reduced NK cell binding to ICAM-1 beads to background levels (**Fig. 3-5D**). These results demonstrate that LFA-1 adhesion to ICAM-1 can be up-regulated on normal NK cells, and by a naturally expressed activating Ly-49 receptor.

D. Discussion

In this study we have shown that engagement of activating Ly-49 molecules stimulates NK cell binding to ICAM-1. Triggering LFA-1 adhesion by DAP-12 coupled receptors is likely to play an important role in how NK cells recognize target cells. Our demonstration of activating Ly-49s triggering LFA-1 binding adds a new dimension to our understanding of how these receptors stimulate cytotoxicity. NK cell LFA-1 binding to target cell ICAM-1 is known to be an important and likely early event in NK cell recognition of a target cell (21). Activating Ly-49 engagement may precede or more likely follow initial low affinity LFA-1 interactions with ICAM-1, but once engaged, activating Ly-49 then significantly up-regulate LFA-1 binding to ICAM-1. In turn, the substantially strengthened interaction with target cells will greatly enhance opportunities for increased interaction of activating Ly-49 and other low affinity activating NK cell receptors to engage their ligands, amplifying NK cell activation signals. Furthermore, LFA-1 engagement by ICAM-1 is known to induce cytolytic granule polarization toward target cells (21). Up-regulation of LFA-1 binding to ICAM-1 through activating Ly-49 engagement may also promote this event, which combined with amplified activation signals, may lead to rapid target cell cytotoxicity. Thus, activating Ly-49 receptors may act as sensors to amplify signals for adhesion and activation.

It is well established that multiple distinct receptors expressed on NK cells cooperate to induce cytolytic function and release of cytokines (7, 21, 35, 36). In fact, resting human *ex vivo* NK cells appear to require engagement of at least two independent receptors to become activated (37). These same investigators also demonstrated that when LFA-3 or CD48, ligands for CD2 and 2B4, respectively, are co-expressed on target cells with ICAM-1, a ligand for LFA-1, this led to enhanced adhesion of resting NK cells, significantly beyond that observed to target cells expressing only ICAM-1 (38). Furthermore, in the case of LFA-1 expressed on human NK cells, it also can physically associate with DNAM-1, another adhesion and signaling receptor and regulate its phosphorylation state and ability to induce NK cell cytotoxicity (39). Although NK cell receptors clearly

cooperate and may even associate, it has not previously been demonstrated that one defined NK activating cell receptor can regulate the adhesive function of another NK cell receptor for its ligand.

Human NK cells can bind ICAM-1 directly with no direct stimulus, albeit to a much lesser extent for resting human NK cells, therefore it has been proposed that NK cells may be unique (38), and unlike T cells, in not requiring stimulation through an independent activating receptor before LFA-1 can bind tightly to ICAM-1. We did not observe significant binding of resting mouse NK cells or the rat RNK-16 cell to ICAM-1 in the absence of stimulation via an activating receptor. We do not know why there are apparent differences in regulation and/or activation state of human and mouse LFA-1 on NK cells, however, our results show that LFA-1 adhesion can be up-regulated by mouse Ly-49 activating NK cell receptors, similar to LFA-1 regulation by T cells. It remains to be determined whether LFA-1 adhesion is regulated in a similar fashion on resting human NK cells. However, given that LFA-1 mediated adhesion in human NK cells can be negatively regulated by inhibitory receptors, it seems likely that LFA-1 adhesion is modulated in human NK cells as well (30, 40).

We showed a requirement for DAP12 association in activating Ly-49 regulation of LFA-1 ligand binding activity, indicating that ITAM-dependent signals are initiating Ly-49 triggered "inside-out" signaling for LFA-1 regulation. Regulation of LFA-1 adhesion is a complex process and Ly-49 may alter LFA-1 avidity through changes in LFA-1 membrane mobility or the affinity of LFA-1 for ICAM-1 (41). Signaling for LFA-1 regulation in lymphocytes is only partially understood, but a few key components known to be involved in TCR-induced LFA-1 regulation are also expressed by mouse and rat NK cells, e.g. LAT (42, 43), SLP-76 (44) and ADAP(45). TCR signaling uses LAT to recruit and activate SLP-76 thereby linking to ADAP, with the latter specifically required for LFA-1 clustering (reviewed in (46)). The Ly-49-DAP12 receptor complex in NK cells employs LAT, but in the absence of LAT, the adapter LAB, which is not expressed in T cells, is recruited to trigger cytotoxicity (47). Given that LAT and LAB have some overlapping but many distinct activities, NK cells may have

redundancy or greater flexibility in LFA-1 regulation, compared to T cells, which may exclusively use LAT. Our data indicate that Ly-49-induced regulation of LFA-1 binding to ICAM-1 requires SFK and Syk tyrosine kinase activities as the binding of RNK-P cells to ICAM-1 was effectively inhibited with doses of PP2 and piceatannol within their respective IC_{50} values (2 and 10 μ M, respectively) in RNK-16 cells. We observe a modest increase in binding to ICAM-1 using drug doses lower than the IC_{50} values for which we presently have no obvious explanation. The Syk and SFKs have been shown to play important roles in the regulation of Ly-49D-dependent responses (14, 48, 49). SFKs have been shown to play a pivotal role in DAP12 phosphorylation (49) and may promote formation and stability of ADAP/SKAP-55/SLP-76 complexes (45, 50, 51). Similarly, SFKs may modulate LFA-1 binding to ICAM-1 following Ly-49/DAP12 induction by stimulating the activation of Syk kinases directly, and RAP-1 indirectly (49, 52, 53). Interestingly, NKG2D also associates with DAP12 and DAP10 in mouse NK cells (54, 55), and it was very recently shown that the NKG2D-dependent elimination of certain tumor cells requires LFA-1 recognition of ICAM-1 (56). It remains to be determined whether NK receptors other than Ly-49s which also associate with ITAM-containing signaling adapters, or receptors such as NKG2D that can associate with distinct signaling adapters, also regulate LFA-1 adhesive properties.

In summary, we reveal a novel aspect of the regulation of NK cell effector functions by demonstrating that activating Ly-49 receptors up-regulate NK cell binding to ICAM-1 through a DAP12-dependent mechanism. The Ly-49 driven mobilization of LFA-1 binding capacity, likely represents an important proximal event leading to the eventual death of a target cell.

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Footnotes:

¹ A version of his chapter has been published in the *Journal of Immunology* (2007), 178 (3): 1261-1267. Copyright 2007. The American Association of Immunologists, Inc.

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E. Individual Contributions: All experimental studies, with the exception of cloning the wild type Ly-49P^{NOD} receptor and the purification of mouse ICAM-1, were conducted by M. Osman. Cloning of the Ly-49P^{NOD} receptor was conducted by Dr. E. Silver, and purification of mouse ICAM-1 was performed by A. Kokaji. M. Osman wrote and updated the paper with modifications provided by Dr. D. Burshtyn and Dr. K. Kane.

³**Abbreviations:** ⁵¹Cr, Na⁵¹CrO₄; FSC, forward scatter; g α m, goat anti-mouse secondary Ab; g α r, goat anti-rat secondary Ab; SSC, side scatter.

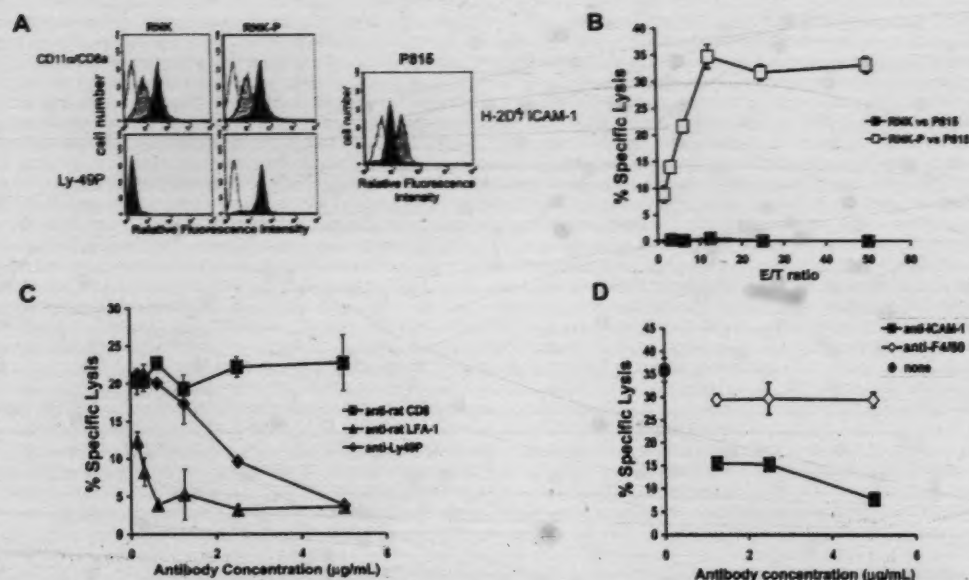


Figure 3-1. Ly-49P and LFA-1 control cytolysis by RNK-P cells. (A) Expression of receptors and ligands on RNK-16, RNK-P and P815 cells. RNK-16 or RNK-P cells were stained with α -rat CD11a (dark gray), α -rat CD8 α (light gray) (upper panels) or α -Ly-49P (black, lower panels). P815 cells were stained for H-2D^d (light gray), or ICAM-1 (black) (far right panel). Corresponding isotype controls are unshaded. (B) RNK-16 or RNK-P mediated cytotoxicity was measured by ^{51}Cr -release from P815 labeled target cells. (C, D) Ly-49 and LFA-1 both contribute to RNK-P lysis of P815 cells. Cytotoxicity was measured in the presence or absence of the indicated antibody-protein A/G complexes at an E/T ratio of 12.5/1. Standard deviation for each triplicate is indicated by the error bars.

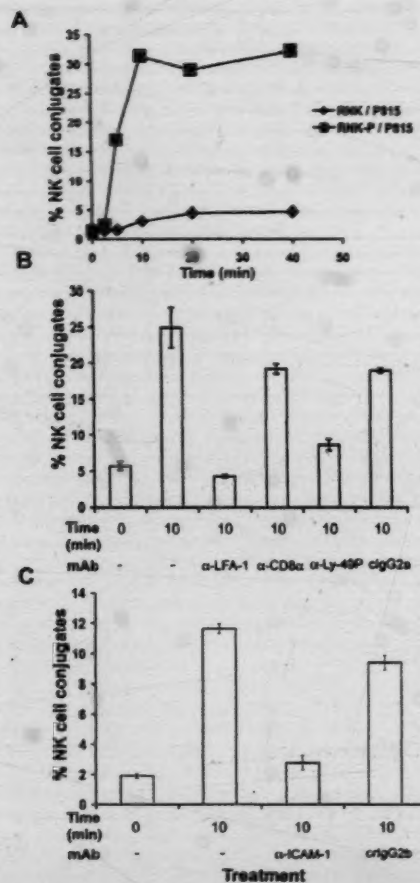


Figure 3-2. Ly-49P regulates adhesion of RNK cells to P815 cells in a LFA-1/ICAM-1 dependent manner. (A) RNK or RNK-P cells were labeled with a red membrane linker dye, PKH26, then incubated with P815 cells labeled with the green dye, PKH67, for the indicated times at an E/T of 1/2. The percentage of NK cells in conjugates was determined using two color flow cytometry. (B, C) Ly-49P facilitates tight adhesion to target cells that is LFA-1/ICAM-1 dependent. Anti-LFA-1 (1.25 μ g/mL), anti-Ly-49P (A1) (2.5 μ g/mL), control anti-CD8 α (2.5 μ g/mL) or isotype control (B27M1, 1.25 μ g/mL) antibodies were pre-incubated with RNK-P cells. Alternatively, anti-ICAM-1 or anti-F4/80 (5 μ g/ml) were incubated with P815 cells pre-incubated with 2.4G2 (10 μ g/ml). Conjugate assays were conducted at the 10 min time point. For samples analyzed at the 0 min time point, cells were immediately fixed then analyzed for heteroconjugates. All panels are representative of at least three independent experiments. Results represent mean $\pm$ SD of triplicate samples.

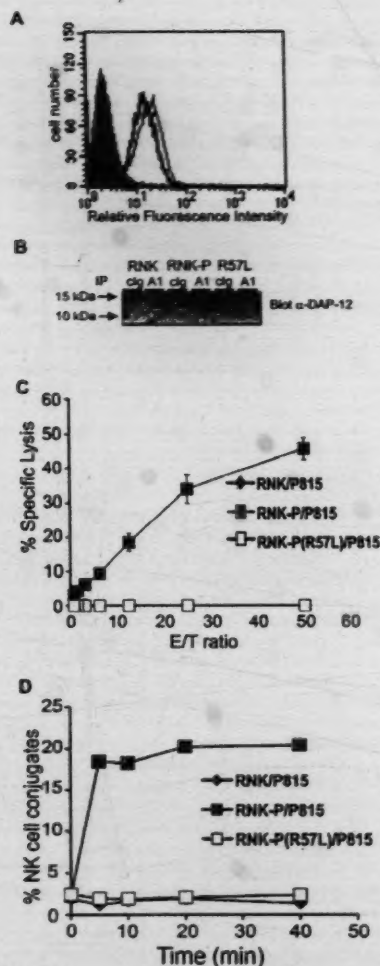


Figure 3-3. DAP12 association with Ly-49P is required for RNK-P cell adhesion to P815 target cells. (A) RNK-P or RNK-P(R57L) cells were stained with A1 (black and gray lines, respectively), or isotype matched control (solid) followed by secondary rat anti-mouse antibodies. (B) DAP12 association with Ly-49P but not Ly-49P(R57L). Pre-cleared cell lysates were immunoprecipitated using A1 or isotype control (B27M1), and blotted with an anti-DAP12 serum. (C) Cytolysis of ^{51}Cr -labeled P815 targets and (D) adhesion to P815 targets are induced by Ly-49P but not Ly-49P(R57L). Standard deviation for each triplicate is indicated by the error bars. Percent NK cell conjugates was determined at an E/T of 1/2 using two color flow cytometry for the indicated incubation periods. Results are representative of three independent experiments.

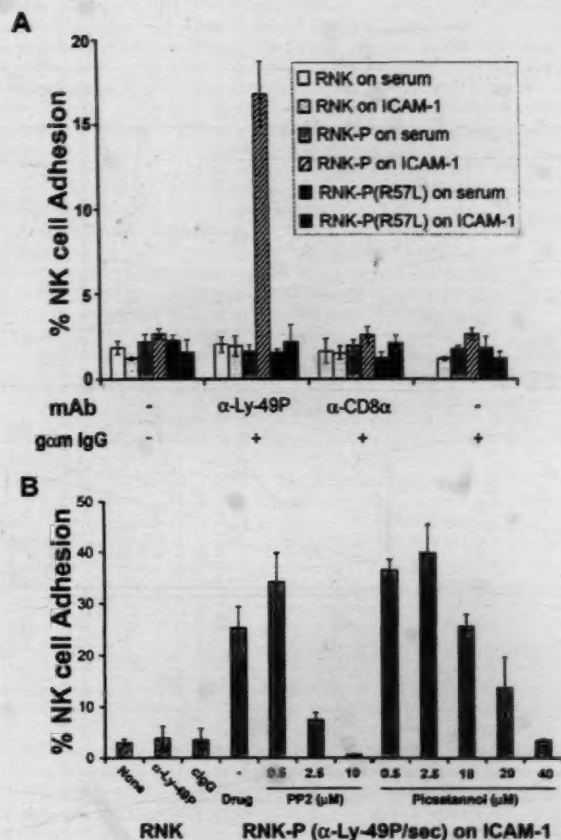


Figure 3-4. Cross-linking of Ly-49P leads to DAP12 and Src/Syk kinase dependent upregulation of LFA-1 binding to isolated ICAM-1. (A) RNK-16, RNK-P, or RNK-P(R57L) were pre-labeled with ^{51}Cr and the FcRs were blocked with 10 $\mu\text{g}/\text{ml}$ of normal rat serum. α -Ly-49P (A1), or α -CD8 α mAbs (5 $\mu\text{g}/\text{ml}$) were mixed with the cells which were then added to the wells immobilized with mouse ICAM-1 (1 $\mu\text{g}/\text{mL}$), or 2% FCS, containing goat anti-mouse secondary (0.5 $\mu\text{g}/\text{mL}$), as indicated. (B) RNK, or RNK-P cells were incubated for 30 min at 37°C in the absence or presence of PP2 or piceatannol at the indicated concentrations, and then used as in (A). Results represent mean $\pm$ SD of each triplicate, and are representative of three or two independent experiments, respectively.

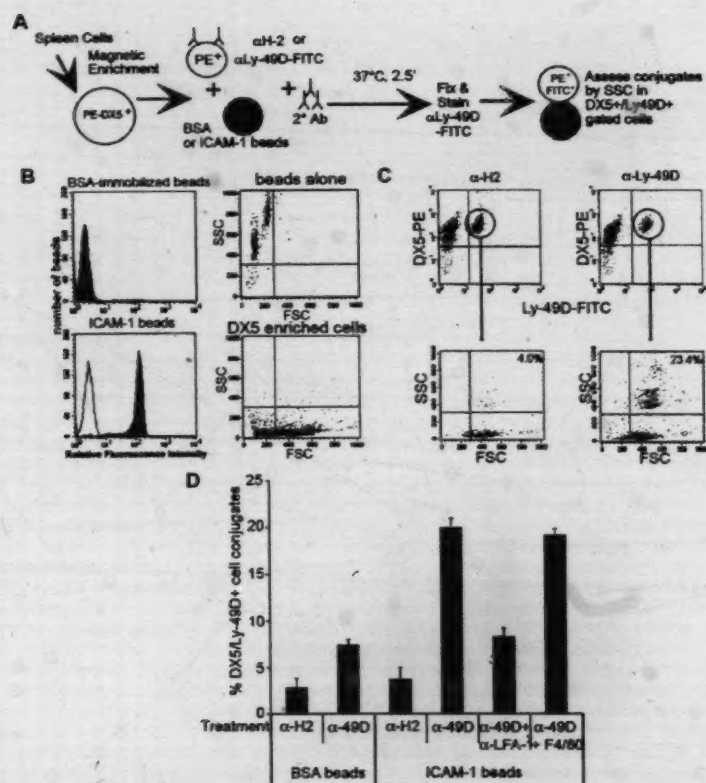
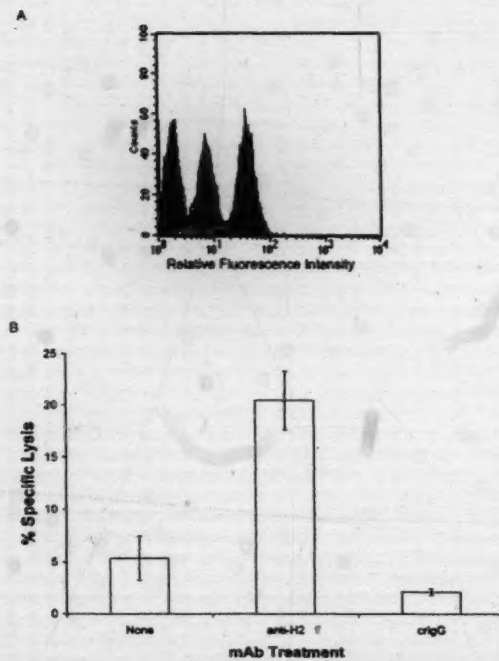
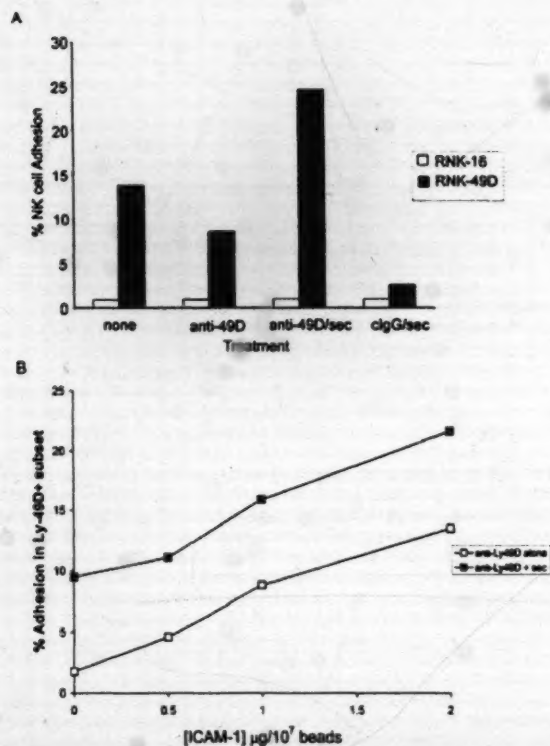


Figure 3-5. Cross-linking of Ly-49D induces *ex vivo* NK cells to bind ICAM-1 via LFA-1. (A) Procedure to measure Ly-49D-induced LFA-1 binding to isolated ICAM-1 with primary cells. Erythrocyte-depleted splenocytes from C57BL/6 mice were enriched for DX5⁺ cells, then treated as indicated. (B) ICAM-1-immobilized beads express a uniform level of ICAM-1, and have a distinct SSC profile from cells. Cell-sized sepharose beads were either immobilized with BSA (top left panel), or mouse ICAM-1 (bottom left panel), blocked with BSA, then stained for mouse ICAM-1 using specific anti-mouse ICAM-1 (solid) or isotype control (unshaded) mAbs, followed by staining with secondary FITC-goat mAbs. FSC and SSC profiles of beads (top right) or cells (bottom right). (C, D) Ly-49D crosslinking results in an LFA-1 dependent increase in cellular adhesion to ICAM-1 beads. DX5-enriched cells were treated as outlined in A, then the percent adhesion of DX5⁺/Ly-49D⁺ cells was determined by first gating on the PE/FITC positive cells as illustrated in panel C, then calculating the percent of cell/bead hetero-conjugates based on FSC/SSC histogram profiles in the presence or absence of α-LFA-1(M17/5.2) or isotype control (F4/80) mAbs (5μg/ml). Data in 5D represent the mean percent cell binding ± SD of triplicate samples. Results in 5C and D are representative of three or two independent experiments, respectively.



Supplementary Figure 3-1. Ly49P(R57L) expresses CD8 α and CD11a and can perform ADCC. A) RNK-P(R57L) cells stained with α -rat CD11a (dark gray), α -rat CD8 α (light gray), or cIgG (black). B) Cytolysis of EL-4 mouse thymoma through ADCC RNK-P(R57L) effector cells in a ^{51}Cr -release cytotoxicity assay and either α H2 (M1/42) or cIgG (Y13) at 5 $\mu\text{g/mL}$.



Supplementary Figure 3-2. Increased ICAM-1 binding by Ly-49D requires primary mAb crosslinking by secondary antibodies. (A) Untransfected RNK-16 cells or cells stably transfected with Ly-49D were assessed for their abilities to bind to ICAM-1-immobilized wells as described in the *Materials and Methods* section in the presence of anti-Ly49D (4E5), cIgG (Y13) alone or in conjunction with γ ar secondary mAb. (B) The percentage of DX5-enriched *ex vivo* NK cell adhesion to BSA or ICAM-1 (as indicated) immobilized beads was determined as described in Fig. 3-5 using anti-Ly-49D (4E5) in the presence or absence of γ ar mAb.

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Chapter 4 - Multiple NK Activating Receptors Regulate LFA-1 Adhesion by *ex vivo* NK cells¹

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A. Introduction

Natural killer (NK) cells play a prominent role in innate immunity by virtue of their ability to recognize and eliminate tumor cells or virally infected cells, while remaining inert to normal self cells (1-3). NK cells confer their protective functions through either the release of cytokines which modulate the immune response, or by directly destroying 'altered' cells through cytotoxic mechanisms (4, 5). Activities of NK cells are regulated by the net signal of combined inputs generated from activating, and inhibitory receptors (1).

NK cells express a variety of activating receptors that regulate their functions. In mice, NK activating receptors are either members of immunoglobulin (Ig) superfamily of receptors such as CD16 – the receptor responsible for antibody dependent cellular cytotoxicity (ADCC), or the C-type lectin family of receptors which include NKG2D and activating members of the NKRP-1 or Ly-49 receptor families. CD16, or Fc γ RIII, binds immune complexes with a low affinity (6). In mice, NK cells and NKT cells are the only lymphoid cells that express this receptor (6-8). CD16 does not have an intrinsic signaling capacity. Instead, it associates with the immunoreceptor tyrosine-based activation motif (ITAM) containing FcR- γ signaling adapter through an intramembrane electrostatic interaction (6). FcR- γ is a disulfide-bonded homodimer that contains an ITAM on the cytoplasmic tail of each subunit (9).

Like CD16, activating members of the NKRP-1 family associate with the signaling adapter FcR- γ in order to mediate their NK functional effects (10). NKRP-1 family receptors are C-type lectins that are type II homodimeric membrane glycoproteins. This family includes the activating receptor NKRP-1C (NK1.1) (11) originally identified as a marker for NK and NKT cells in C57BL/6 mice (12, 13). The ligand for NKRP-1C has not been clearly defined. However, the responses of NK cells to NKRP-1C ligation are similar to that of CD16, as the

cross-linking of the receptor by mAb ultimately leads to NK cytokine release or the polarized exocytosis of cytotoxic granules (10).

NKG2D is another member of the C-type lectin receptor family of NK receptors. It recognizes members of the retinoic acid early transcript (RAE) family of tumor associated antigens, or the MHC-like antigens H-60 and Mult1 (14, 15). Similar to NKRP-1C, NKG2D is expressed as a disulfide-linked homodimer, on the surface of NK cells and some T cells. In mice, NKG2D is expressed as two isoforms. Unstimulated NK cells express a full length form of the receptor (NKG2D-L), while IL-2, or poly I:C² activated cells express the NKG2D-L form or a shorter form (NKG2D-S) that results from alternate splicing. NKG2D-L exclusively associates with the homodimeric signaling DAP10 adapter to mediate its activating receptor effector functions, while NKG2D-S associates with either DAP10 or another disulfide-bonded ITAM-containing homodimeric signaling adapter, DAP12 (16, 17). DAP10, unlike FcR- γ or DAP12, does not employ ITAMs to initiate biochemical signaling cascades. Instead, it contains a YxNM motif that uses phosphoinositide-3-kinase (PI3K) as its primary signaling effector molecule (18).

Activating Ly49 receptors are also disulfide-linked homodimeric glycoproteins (19). Their association with the signaling adapter DAP12 is important and essential for their induction of NK cell cytotoxicity or cytokine responses (20-22). Activating Ly-49 receptors play an integral role in conferring immunity to MCMV and in the destruction of tumor cells by recognizing MHC I or MHC I-like ligands (23-25). For example, Ly-49H directly recognizes the mouse cytomegalovirus (MCMV) MHC I-like m157 gene product, while Ly-49P confers immunity to MCMV through an H-2D^k-dependent mechanism in mouse strains not expressing Ly-49H (25, 26). Likewise, Ly-49D recognizes H-2D^d, and a xenogeneic ligand on chinese hamster ovary (CHO) tumor cells (23, 27).

NK cells also express LFA-1 ($\alpha_L\beta_2$), a β_2 -integrin that recognizes ICAM-1-5, which is important for regulating adhesion to target cells (28-30). In various cell types, signals emanating from activating receptors regulate the affinity and/or avidity of LFA-1, ultimately resulting in increased binding of ICAM-1 (31-33).

LFA-1-mediated tight adhesion to target cells stabilizes the intercellular adhesion between cytotoxic cells and their targets thereby facilitating the delivery of NK cytotoxic granules to target cells (28, 34). Inside-out signaling producing firm LFA-1 mediated adhesion, for example, between cytotoxic T cells and their targets, is triggered by signaling resulting from TCR engagement (32). Although NK receptors that associate with ITAM-containing signaling adapters share some similarities with the TCR, such as involvement of an ITAM and Syk family kinases in transmembrane signaling, it has only recently been demonstrated that activating Ly-49 receptors regulate LFA-1 adhesion through inside-out signaling (35). In addition, the properties of activating Ly-49-dependent activation of LFA-1 binding to ICAM-1 have not been closely examined. Furthermore, the role NKG2D plays in this process has not been established.

In this study we show that CD16, NKG2D, NKRP-1C and Ly-49D receptors all can promote adhesion of NK cells to ICAM-1 in an LFA-1-dependent mechanism. We also show that the crosslinked Ly-49D receptor exhibited different kinetics and sensitivity to ICAM-1 compared to other activating receptors. Together, these results highlight the role activating NK receptors play in regulating adhesion to target cells through LFA-1.

B. Materials and Methods

Animals and injections

Female C57BL/6 mice at 6–8 wk of age were purchased from the Jackson Laboratory (Bar Harbor, ME). For *in vivo* injections, mice were injected i.p. with 200 µg of poly I:C in PBS (1mg/mL) (Sigma-Aldrich, Oakville, ON) or sterile-filtered phosphate buffered saline (PBS). After 20 h, mice were euthanized and the spleens were harvested. Experiments were approved by the Animal Welfare and Policy Committee of the University of Alberta (Edmonton, Alberta, Canada).

NK cell purification, cell lines and antibodies

NK cells were prepared using the Easy Sep DX5⁺ selection kit (Stem Cell Technologies; Vancouver, BC) from RBC-depleted single cell suspensions of C57BL/6 spleens. Chinese hamster ovary cells (CHO), Daudi human Burkitt's lymphoma cells, and YAC-1 mouse T lymphoma cells were obtained from American Type Culture Collection. YAC-1 cells were maintained in RPMI 1640 supplemented with 10 % FCS, while Daudi and CHO cells were maintained in DMEM supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The following antibodies were produced from the hybridomas as described (36): YN-1/1.7.4, anti-mouse ICAM-1 (rat IgG2b) (37); M1/42, anti-mouse H-2 (rat IgG2a) (38); (Y13), anti-rat p21^{ras} (rat IgG2a) (37), 2.4G2 (rat IgG2b) anti-mouse Fc-γ receptor (39); PK136, anti-NK1.1 (mouse IgG2a) (40), 28-14-8S anti-H2D^b α3 region (41); B27M1, anti-HLA-B27 (mouse IgG2a) (42); M17/5.2, anti-mouse LFA-1 (rat IgG2b) (43), and anti-mouse F4/80 antigen (rat IgG2b) (44). Functional grade MI-6, anti-mouse NKG2D (rat IgG2a); 93, anti-mouse CD16/32 (rat IgG2a), anti-mouse NKG2D (rat IgG2b), biotin-conjugated M17/4, anti-mouse LFA-1; and streptavidin-APC were purchased from e-Bioscience (San Diego, CA). In addition, purified or FITC-conjugated 4E5, anti-Ly49D (rat IgG2a), was purchased from BD/PharMingen (San Diego, CA). MI-6, PK136, 93 mAbs and Y13 were directly coupled to Alexa-Fluor® 488, using the manufacturer's instructions. FITC-coupled rat anti-mouse IgG or mouse anti-rat IgG were

purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified γ m, γ r, and unmodified rat IgG were purchased from Sigma-Aldrich (Oakville, ON).

Flow Cytometry

For the detection of cell surface receptors, DX5⁺ cells were pre-incubated with 5 μ g/mL of 2.4G2 (unless CD16 was being measured) for 15 min at RT then the indicated FITC conjugated mAb was added (2 μ g/mL) for 30-45 min on ice. Following the incubation, the cells were washed two times using PBS, were then fixed with 2% *p*-formaldehyde in PBS, then analyzed using a FACSCalibur® flow cytometer. For the detection of cell surface CD16, single color analysis was used using 2.4G2-FITC (for CD16) or Y13-FITC (cIgG) and the percentage of CD16 positive cells was determined. Unless otherwise indicated, the frequency of expression for other receptors was determined using two-color flow cytometry. For the detection of cell surface CD11a, biotin conjugated anti-mouse LFA-1 antibodies (1 μ g/mL) were added to cells for 30 min on ice. Subsequently, the cells were washed two times using PBS, then streptavidin-APC was added to the cells for 15 min on ice, then the cells were washed two additional times before their analysis using a FACSCalibur® flow cytometer.

Purification of mouse ICAM-1 and the immobilization of ICAM-1 on cell-sized beads

Briefly, mouse ICAM-1 (mICAM-1) was affinity-purified from mouse A20 cells using a YN-1/1.7.4 gravity sepharose column as described (45). Following the collection of sequential 1 mL fractions, each fraction was measured for total protein content using a microBCA kit (Amersham Biosciences, NJ) and for ICAM-1 bioactivity via solid-phase ELISA with YN-1/1.7.4 as the primary antibody, and goat anti-rat-horse radish peroxidase (Jackson Immunoresearch) for detection. Following its purification, mICAM-1 was immobilized to cell-sized latex beads, by dilution in PBS, then adding 10^7 diluted beads to the diluted

ICAM-1 with rotation for 30-45 min at 4° C. Subsequently, unbound sites on the cell-sized beads were blocked with 2 % bovine serum albumin (BSA) in PBS at 4° C overnight.

Cytotoxicity assays

Ex vivo fresh or poly I:C activated DX5-enriched NK cells were used as effector cells for all cytotoxicity assays. Target cells were labeled at 37°C with 100 µCi of Na⁵¹CrO₄ for 1-2h. Following extensive washing, 1×10^4 ⁵¹Cr-labeled targets were incubated for 4 h at 37°C in V-bottom microtiter plates at various E/T ratios in triplicate as described (36). For redirected lysis assays, FcR-expressing Daudi cells labeled with ⁵¹Cr were pre-incubated for 15 min with 5 µg/ml of MI-6, Y13, 2.4G2, F4/80, PK136 or B27M1 mAbs in RPMI-1640 media, then *ex vivo* DX5-enriched NK cells expressing the receptors recognized by the specific mAb (effector to target ratio (E/T) 6/1) were co-incubated for 4 h and cytotoxicity was measured as described above.

Conjugate assays

Adhesion assays with *ex vivo* NK cells were conducted as previously described (35). Briefly, DX5⁺ cells were treated with α-Ly-49D-FITC, α-CD16 (clone 93 (rIgG2a), or 2.4G2 (rIgG2b)), α-NKG2D (clone MI-6), α-NK1.1, α-H2 mAbs (M1/42 or 28-14-8S) or F4/80 at $1\mu\text{g}/10^6$ cells for 5 min at room temperature, then mixed with cell-size beads with immobilized BSA or ICAM-1 (ICAM-1 concentrations as indicated) in PBS containing 1.0 mM MgCl₂ and 0.9 mM CaCl₂ at an effector/bead ratio of 3/2, then centrifuged at 30 x g for 3 min. Cell/bead mixtures treated with mAbs were cross-linked using γar or γam (0.5 µg/mL) for 0 to 120 min at 37 ° C (as indicated), then fixed with 1% *p*-formaldehyde. Ly-49D⁺ cell conjugates were determined as the percentage of DX5⁺/Ly49D⁺ cells shifted to a SSC corresponding to that of the beads as previously described (35). Similarly, cross-linking studies using CD16, NK1.1, or NKG2D, cell conjugates

were determined as the percentage of DX5⁺-PE cells shifted to a SSC profile of the beads.

C. Results

Ex vivo NK cells regulate LFA-1 adhesion to ICAM-1 via Ly-49D, CD16, NKG2D and NKRP-1C

We had previously demonstrated that activating Ly-49 regulate adhesion to ICAM-1 by *ex vivo* NK cells (35). Because Ly-49D is only expressed on ~ 27 % of DX5⁺ NK cells (**Fig. 4-1A**), we employed the use of a single cell flow cytometric bead-based adhesion assay to measure the adhesion of the Ly-49D⁺ subset of NK cells following soluble mAb crosslinking of the receptor (35). The Ly-49D dependent adhesion to ICAM-1 detected was LFA-1 dependent, as blocking mAbs to the LFA-1 CD11a subunit substantially reduced the level of adhesion (35). To understand whether other activating receptors non-covalently associating with other signaling adapters also regulate LFA-1-specific adhesion, we adapted a similar strategy as that employed for Ly-49D and examined CD16, NKRP-1C, and NKG2D. Unlike Ly-49D, these receptors are expressed on a large subset of DX5⁺ NK cells. In fact, CD16 is expressed on over 85 % of enriched NK cells (based on single color flow cytometry using CD16-FITC), and the frequency of both the NKRP-1C and NKG2D expressing NK cells was over 70 % **Fig. 4-1A**. In addition, the expression level of these receptors on *ex vivo* cells, albeit based on staining with different antibodies, appeared to be lower than that observed for Ly-49D (**Fig. 4-1A**). In contrast, LFA-1 on *ex vivo* NK cells was uniformly present at high levels (**Fig. 4-1A**).

Upon crosslinking with mAbs recognizing the individual receptors, an increase in the number of DX5⁺ NK cells adhering to ICAM-1 beads is observed only with the antibodies recognizing the NK receptors but not isotype matched control antibodies (**Fig 4-1B**). Moreover, the adhesion is specific for the interaction of ICAM-1 on the beads with LFA-1, as little NK cell binding is observed with BSA beads and blocking mAb to LFA-1 completely inhibits the NK cell adhesion, whereas corresponding isotype matched control antibodies do not block (**Fig. 4-1B**). These results indicate that engagement of a number of

other NK cell activating receptors of distinct families or subfamilies are also capable of inducing LFA-1 adhesion to ICAM-1, including CD16, NKG2D and NKRP-1C, in addition to activating Ly-49 receptors.

LFA-1 is more responsive at lower ICAM-1 densities by Ly-49D-induced adhesion than NKG2D, NKRP-1C or CD16-dependent adhesion

We next examined the increase in NK cell adhesion induced by the individual activating NK receptors, using ICAM-1 beads immobilized with increasing concentrations of purified mouse ICAM-1. To determine the relative density of ICAM-1 immobilized on the cell-sized beads, the mean fluorescent intensities of ICAM-1 beads was measured using anti-ICAM-1 mAb and flow cytometry. As shown in Fig. 2A, ICAM-1 was efficiently immobilized onto the cell-sized beads at homogeneous densities for each amount of ICAM-1 (**Fig. 4-2A**). In addition, the increase in mean fluorescence intensity of antibody binding to ICAM-1 was directly proportional to the amounts incubated with the beads (**Fig. 4-2B**).

Upon close examination of NK cell adhesion to beads immobilized with different amounts of purified mouse ICAM-1, the maximal levels of NK cell binding induced by Ly-49D ligation appeared to be profoundly higher than that observed for the other receptors. The frequency of Ly-49D⁺ NK cells bound to ICAM-1 beads increased linearly with higher amounts of immobilized ICAM-1 (**Fig. 4-2C**). This direct relationship was maintained until 2.0 µg ICAM-1 beads were used; however, it was not at higher ICAM-1 concentrations suggesting that the physiological response induced by this receptor complex was reaching its saturation point (**Fig. 4-2C**). In contrast to Ly-49D, the frequency of adhesion induced following CD16 and NKG2D crosslinking was only detectable when 2 µg of ICAM-1 were immobilized on the beads (**Fig. 4-2C**), whereas a detectable increase in adhesion following NKRP-1C crosslinking was evident with 0.5 µg ICAM-1 beads (**Fig. 4-2D**).

Interestingly, Ly-49D-induced triggered binding was substantially detected with beads immobilized with as little as 0.25 μ g ICAM-1 (**Fig. 4-2E**) and increased when beads with higher amounts of bound ICAM-1 were used when compared to the isotype matched control. In fact, a detectable frequency of adhesion was only evident when 2 μ g ICAM-1 beads were used with cells crosslinked with NKG2D and CD16. Together, it can be suggested that the ability of the Ly-49D/DAP12 complex is more potent in regulating LFA-1-dependent adhesion to ICAM-1 than the CD16/FcR- γ , NKG2D-L/DAP10, or NKRP-1C/FcR- γ receptor complexes.

Ly-49D/DAP12 triggered binding via LFA-1 is kinetically distinct from that induced by CD16, NKG2D, and NKRP-1C

Studies using T cells have clearly demonstrated that the TCR/CD3 complex induces a rapid, but transient upregulation of LFA-1 adhesion to ICAM-1, lasting approximately 30 min (32). In order to understand whether the various NK activating receptor complexes exhibited similar properties, we examined their LFA-1 induced adhesion over time using beads immobilized with 2 μ g of ICAM-1. Ly-49D stimulation rapidly induced a very rapid (peak at 3 min) and substantial increase in adhesion that was transient (**Fig. 4-3A**). In fact, the LFA-1-dependent Ly-49D-induced adhesion was reduced to background levels within 30 min following its stimulus (**Fig. 4-3A**), suggesting that the upregulation in LFA-1 triggered by Ly-49D/DAP12 was similar in its properties as the TCR. In contrast, the kinetics of CD16, NKG2D and NKRP-1C triggered LFA-1 binding were very different. In the presence of the ICAM-1-immobilized beads, there was a profound increase in LFA-1 mediated NK cell adhesion from background levels; however, this increase was not altered over time when triggered by CD16, NKG2D (**Fig. 4-3B**), or NKRP-1C (**Fig. 4-3C**). In fact, the adhesion levels induced by these receptors following antibody crosslinking were comparable to those measured with Ly-49D that were in decline after 30 min. Together, these results suggest that Ly-49D and the TCR induced LFA-1 binding share common transient kinetics and possibly a similar mechanism, while CD16, NKG2D, and NKRP-1C induced LFA-1 binding is distinct kinetically and long lasting.

Ly-49D, CD16, and NKRP-1C but not NKG2D receptor complexes can trigger a cytotoxic response against tumor targets using ex vivo NK cells

Studies using unstimulated human NK cells have suggested that not all activating receptors can trigger NK cytotoxic or cytokine responses. For example, crosslinking NKG2D in a redirected lysis assay does not result in the cytolysis of P815 targets (46). To determine the activating receptor-induced cytotoxic capacity of Ly-49D, CD16, NKG2D, and NKRP-1C on *ex vivo* mouse NK cells, the cytolysis of mouse YAC-1 lymphoma cells (for natural cytotoxicity), CHO cells (for Ly-49D-dependent xenorecognition of a ligand expressed on the surface of these cells) (27), or redirected lysis using FcR⁺ Daudi cells and mAbs for NKG2D, CD16, NKRP-1C or isotype matched controls was conducted. Upon the engagement of YAC-1 and CHO cells were efficiently lysed by *ex vivo* NK cells (Fig. 4-4A). In the redirected lysis assays, FcR⁺ target cells labeled with ⁵¹Cr were pre-incubated with mAb specific to activating receptors expressed on the surfaces of effector NK cells. Upon their co-incubation with *ex vivo* effector cells, activating receptors on NK cells are crosslinked and a cytolytic activation signal was induced. To this end, cytolysis was detected when mAbs for CD16, and NKRP-1C were used in a redirected cytolysis assay, but not with NKG2D or isotype matched controls, as the levels of cytolysis they induced were undetectable (Fig. 4-4B).

Poly I:C activation of NK cells does not regulate LFA-1-mediated adhesion

In mice, NK cells can be activated *in vivo* through an i.p. injection of the artificial TLR 3 agonist poly I:C. Following its injection, NK cells are activated and a large proportion migrate from the spleen and blood into the liver within 24 h (47). In addition, the NK cells remaining in the spleen are highly potent in mediating direct cytotoxicity, and in ADCC (48). It has been suggested that NK cells derived from mice injected with poly I:C are 'primed' or activated by DC-derived IL-15 (48). To test the role of DC priming in regulating NK activating receptor-induced LFA-1-dependent adhesion to ICAM-1, mice were injected with poly I:C

in vivo, then cells were purified from mice either injected with PBS or poly I:C. Following the purification of DX5⁺ cells, it was clear that the numbers of NK cells derived from poly I:C injected mice were substantially lower than those from the control-injected mice. Importantly, the Ly-49D or LFA-1 receptor levels were unaffected (**Fig. 4-5A**). Although the CD16 and NKG2D receptor levels were not examined due to a substantial reduction in the numbers of NK cells purified from mice injected with poly I:C, it has been previously established that NK cells derived from mice injected with poly I:C have enhanced levels of ADCC and express higher levels of NKG2D (17, 49). As predicted, NK cells derived from poly I:C injected mice more efficiently lysed YAC-1 tumor cells (**Fig. 4-5B**). Interestingly, there was no change in the frequencies of Ly-49D, CD16, or NKG2D-induced adhesion to ICAM-1 (2 μ g) immobilized beads as the detectable adhesion was comparable to those observed for control injected mice (**Fig. 4-5C**). Thus, although additional NK priming is important for the destruction of NK targets, it appears that the ability of NK cells to undergo activating receptor induced adhesion to ICAM-1 is unaffected.

D. Discussion

In this study we have shown that crosslinking different individual NK activating receptors by soluble antibodies stimulate mouse *ex vivo* NK cell binding to ICAM-1 immobilized on cell-sized beads in an LFA-1 dependent manner. LFA-1 engagement of ICAM-1 on target cells is an important step in NK-mediated destruction of target cells (34). The ability of multiple NK receptors to regulate LFA-1 adhesion to ICAM-1 suggests that the regulation of tight adhesion may be a global mechanism involved in the regulation of NK effector responses. The modulation of LFA-1 adhesion to ICAM-1 by activating receptors may provide a mechanism to lower the ligand density or receptor/ligand affinity required for an effective response. For example, although the RAE-1 δ NKG2D ligand binds to its receptor with a weak affinity (50), NK cells can still eliminate tumor cells expressing RAE-1 δ *in vivo* (51). In addition, the recruitment of LFA-1 at the site of cell contact can lower the threshold required for T cell antigen receptor-dependent activation by virtue of its interaction with proteins anchored to the actin cytoskeleton (52). Similarly, studies using B cells have suggested a role for the interaction between LFA-1 and ICAM-1 in lowering the threshold required for B cell activation by lowering the level of specific antigen required to form a mature immunological synapse (53).

Our results suggest that different receptors may not have the same capacity or properties in regulating *ex vivo* NK cell adhesion to ICAM-1 expressing cells. For example, the Ly-49D/DAP12 receptor is expressed at higher levels than CD16, NKG2D, and NKRP-1C (Fig. 4-1A), and induces a higher frequency of Ly-49D⁺ NK cell adhesion to ICAM-1 (Fig. 4-2, 3). Unlike the other receptor complexes examined, Ly-49D exclusively associates with DAP12, and this intermolecular association is required for the regulation LFA-1 triggering (35). This receptor complex can mediate adhesion to ICAM-1 at much lower densities than the other receptors; i.e. it sensitizes LFA-1 to low ICAM-1 levels (Fig. 4-2). The enhanced sensitivity to ICAM-1 by activating Ly49 may stem from a direct association of DAP12 with LFA-1. Studies using the NK activating

receptor DNAM-1 have shown that the C-terminal end of the cytoplasmic tail of this receptor contains the conserved sequence SRRPK (between mouse and human). DNAM-1 constitutively associates with LFA-1 in human NK cells, and can inducibly associate with LFA-1 following anti-TCR crosslinking in T cells (54). The association in T cells requires the phosphorylation of the Ser residue in the SRRPK sequence (54). Interestingly, DAP12, but not the DAP10 or FcR- γ signaling adapters, contains a highly conserved homologous sequence, TRKxR/H, eight amino acids upstream of the cytosolic ITAM; however, there is no direct evidence supporting the notion that DAP12 associates with LFA-1 via this sequence.

Alternatively, the increased sensitivity to ICAM-1 induced by the Ly-49D/DAP12 receptor complex may stem from an increased LFA-1 concentration at the site of ICAM-1 contact resulting from increased LFA-1 lateral mobility in response to activating Ly-49 receptor crosslinking. Studies using the TCR have shown that LFA-1 lateral mobility increases following receptor crosslinking (55). The increased lateral mobility evident with the TCR is coupled to an increased LFA-1 affinity for ICAM-1 and results in a robust transient increased binding to ICAM-1 (32). Our results indicate that Ly-49D/DAP12 crosslinking also results in a strong but transient induction of Ly-49D⁺ NK cells adhering to ICAM-1 beads (Fig. 4-3), suggesting that the mechanism employed by this receptor system may resemble that used by the TCR.

Signaling for LFA-1 regulation in lymphocytes is only partially understood, but a few key components known to be involved in TCR-induced LFA-1 regulation are also expressed in murine NK cells, e.g. LAT (56, 57), SLP-76 (58), Vav (59), ADAP (60), Rap1, and Mst1 (61). TCR signaling uses LAT to recruit and activate SLP-76 thereby linking it to ADAP, with the latter specifically required for LFA-1 clustering (reviewed in (62)). The adapter protein RIAM, can in turn, link LAT/SLP-76 signals to Rap1/RapL and Mst1 (61) via ADAP/SKAP55 (63). The contributions of these proteins to Ly-49D/DAP12 triggering of LFA-1 by NK cells is worthy of investigation.

Our data do not support the notion that NK activating receptors other than activating Ly-49 utilize a TCR-like mechanism to regulate adhesion. For example, unlike the TCR or Ly-49D, CD16/FcR- γ and NKRP-1C/FcR- γ or NKG2D/DAP10, generate a sustained lower frequency of NK cell adhesion that is only detectable at higher concentrations of ICAM-1 (Fig. 4-2, 4-3). The low frequency of adhesion evident upon CD16, NKG2D, or NKRP-1C crosslinking may directly stem from the individual receptor complexes as reminiscent of studies illustrating changes in LFA-1 affinity for ICAM-1 in the presence of chemokine stimuli (64). LFA-1 adapts three conformations with low, intermediate or high affinity for ICAM-1 (62). The low affinity form limits its ligand binding domain to a proximal location to the membrane, while the higher affinity forms apparently project the integrin stalk away from the cell membrane (62). The intermediate affinity form can be induced by inside-out signals such as those generated by PI3K (62). This form of LFA-1 can further be stabilized upon ICAM-1 binding (65). Future studies using soluble ICAM-1 molecules (66), may provide some insight regarding the role of FcR- γ or DAP10 receptor complexes in regulating LFA-1 conformation and affinity level.

Activating receptors expressed by human NK cells have been shown to generate synergistic signals (46). In fact, the same authors demonstrated that the NKG2D/DAP10 receptor complex is unable to induce a cytolytic response in unstimulated NK cells, while the CD16 receptor complex is functionally competent (46). Upon activation of the NK cells using IL-2, NKG2D cytolytic responses were generated (46). Our results suggest that *ex vivo* mouse NK cells behave in a similar manner as human unstimulated *ex vivo* NK cells, as Ly49D/DAP12, NKRP-1C/FcR- γ , and CD16/FcR- γ were able to induce the lysis of target cells, but NKG2D-L/DAP10 was not (Fig. 4-4). The relatively low specific lysis measured using *ex vivo* mouse NK cells in redirected lysis by CD16 or NKRP-1C may stem from the low expression of proteins involved in granule mediated cytotoxicity, e.g. perforin, granzyme B, as these NK cells have not been adequately (48). In addition, it may also result from the use of human target cells

expressing human ICAM-1, which may not be optimal for mouse LFA-1 engagement as previously observed (11). Previous studies using *ex vivo* NK cells have suggested that mouse NK cells express functional CD16 and Ly-49D receptor complexes (67, 68), although it was not clear whether they express a functional NKRP-1C receptor complex. Our results suggest that *ex vivo* NK cells express surface CD16, Ly-49D and NKRP-1C receptor complexes capable of generating a cytolytic response. They also suggest that the signals generated by the NKG2D/DAP10 receptor complexes are sufficient to trigger mild changes in LFA-1 binding for ICAM-1, but are not sufficient to mobilize the cytolytic machinery.

Examining possible synergism between multiple NK receptors with respect to increased adhesion and cytotoxicity will be an area of active future research. In fact, the increased background adhesion frequently evident upon crosslinking with the isotype-matched control for anti-NKRP-1C, but lower than those achieved with anti-NKRP-1C, at higher ICAM-1 concentrations indirectly supports this notion (**Fig. 4-2D**). This mAb belongs to the IgG2a isotype which inherently bind with a much higher affinity to CD16 than other mAb isotypes (6) – thereby suggesting that synergistic signals are present between NK1.1 and CD16. As a result, the use of (Fab)₂ forms of PK136 or its isotype matched control will be necessary in future studies to discriminate between the individual contributions of the NKRP-1C and CD16 receptor complexes in regulating LFA-1 adhesion. Synergism between multiple receptors has been shown to play an important role in regulating NK cell functions. For example, DNAM-1 synergizes with natural cytotoxicity receptors to regulate the lysis of dendritic cells (69). Together, these findings are consistent with studies suggesting that resting human *ex vivo* NK cells require the engagement of at least two independent receptors to become activated (46). As NK receptors express an extensive repertoire of activating receptors, the synergy between different receptors may be of significant importance in the regulation of different aspects of NK effector functions.

Recently, an additional dimension in NK cell recognition of target cells has been revealed. Pathogen-activated DC present IL-15 in *trans* to NK cells, and this presentation is required for an effective NK anti-viral response (48). The authors used poly I:C as an artificial TLR agonist to demonstrate that NK cells from a mouse injected with this TLR 3 ligand are more cytolytic. However, primed NK cells do not spontaneously produce IFN- γ or mediate cytotoxicity (48). Our results corroborate this observation as DX5-enriched NK cells from a poly I:C injected mouse can more efficiently lyse YAC-1 tumor target cells than unstimulated control mice (Fig. 4-5B). In addition, our results suggest that NK priming by DCs does not affect the increased adhesion by Ly-49D, NKG2D or CD16 (Fig. 4-5C). Thus, the IL-15-mediated enhanced NK effector functions likely stems from increased gene expression of effector molecules involved in cytotoxicity eg. granzymes and FasL (70). However, studies directly examining the role of IL-15 *trans* presentation in NK cell activating receptor regulated adhesion will be required to understand the role this cytokine plays in this process.

In summary, we demonstrate that multiple NK receptors up-regulate NK cell binding to ICAM-1. We also provide evidence indicating that the nature of LFA-1 binding induced by various NK cells can differ, with that by activating Ly-49 being strong, rapidly induced and transient, while that of other activating NK receptors is less pronounced, yet sustained. Activating NK cell receptor enhancement of LFA-1 binding capacity, likely represents an important proximal event leading to augmented signaling, which ultimately lowers the threshold required for NK activation and destruction of susceptible target cells.

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Footnotes:

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E. Individual Contributions: All experimental studies were conducted by M. Osman. M. Osman wrote and updated the chapter with the modifications suggested by Dr. D. Burshtyn and Dr. K. Kane.

²**Abbreviations:** APC, allophycocyanin; ⁵¹Cr, Na⁵¹CrO₄; FSC, forward scatter; α m, goat anti-mouse secondary Ab; α r, goat anti-rat secondary Ab; i.p., intraperitoneal; ITAM, immunoreceptor tyrosine based activation motif; MFI, mean fluorescence intensity; mICAM-1, mouse intercellular adhesion molecule 1; poly I:C, poly inosinic acid:cytidylic acid; SSC, side scatter.

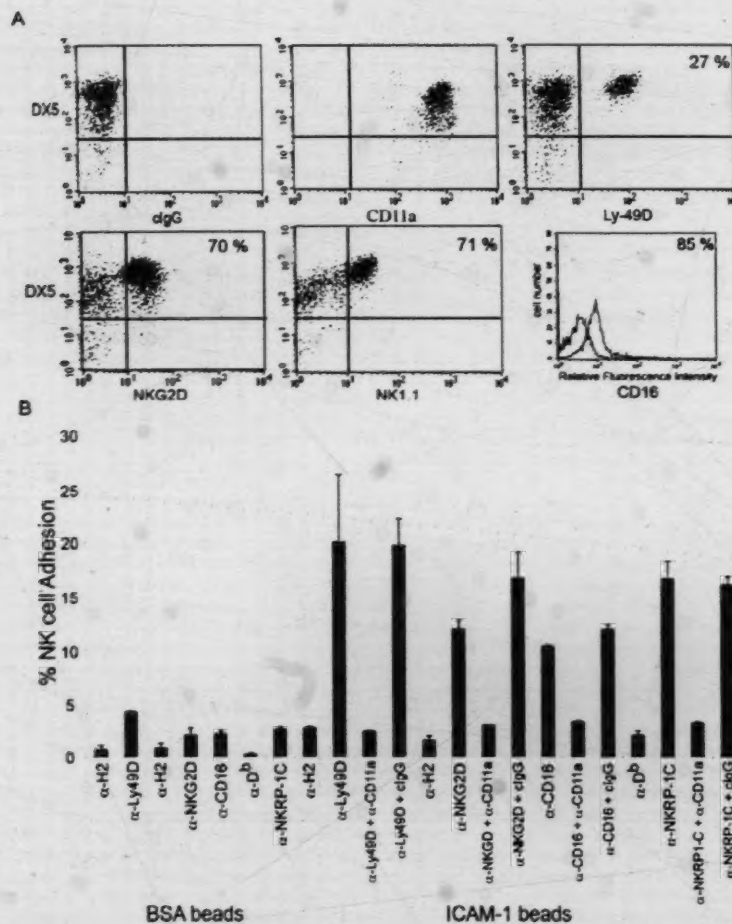


Figure 4-1. Engagement of CD16, NKG2D, NKRP-1C and Ly-49D increases adhesion of NK cells to ICAM-1 in an LFA-1 dependent manner. (A) Expression of CD11a, Ly-49D, NKG2D, CD16, and NKRP-1C (NK1.1) on the surface of *ex vivo* DX5-enriched C57BL/6 NK cells. NK cell FcRs were pre-blocked with 2.4G2 (unless CD16 expression was monitored), and the cell surface expression of the indicated receptors was determined using either two color or single color flow cytometry (for CD16) as outlined in the *Materials and Methods* section. The frequency of receptor expression for each NK activating receptor is indicated in the top right corner of each corresponding plot. (B) NK adhesion to BSA or ICAM-1-immobilized beads ($2 \mu\text{g}/10^7$ beads) was either determined within the Ly-49D subset or for total DX5⁺ cells following the crosslinking of the indicated primary antibodies with $\text{g}\alpha\text{r}$ or $\text{g}\alpha\text{m}$ antibodies in the presence or absence of anti-LFA-1 α chain blocking antibodies or isotype matched controls as described in the *Materials and Methods* section. These results represent two and three independent experiments, for panels A and B respectively.

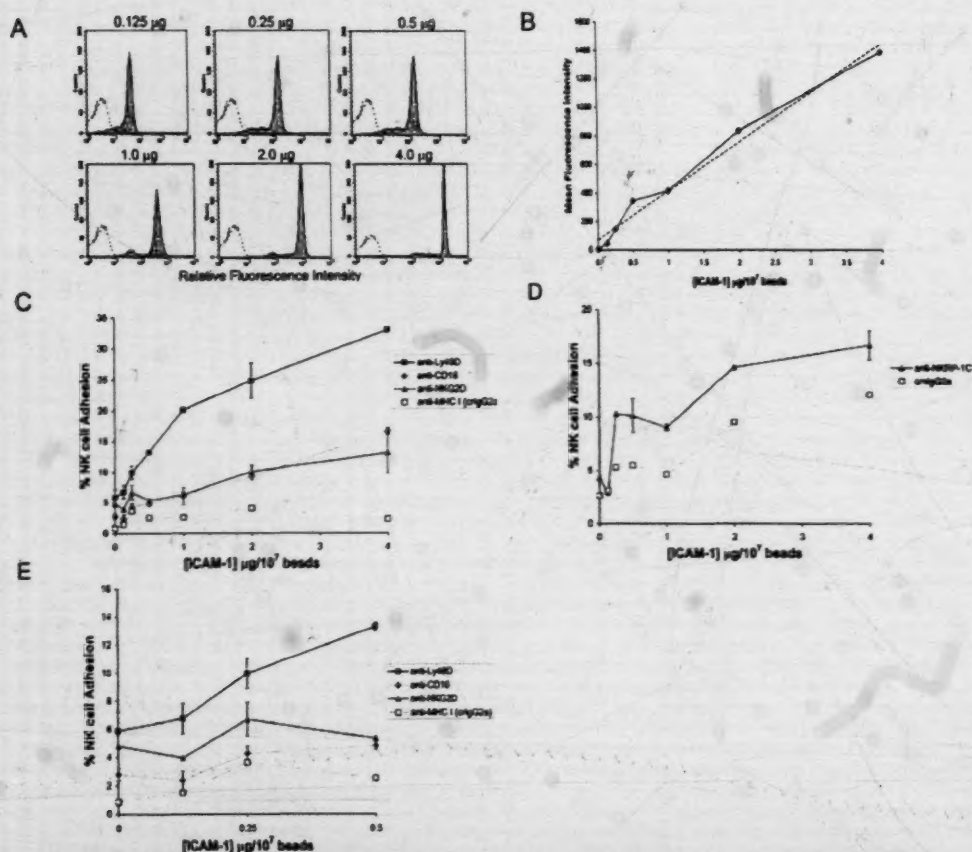


Figure 4-2. CD16, NKG2D, NKRP-1C and Ly-49D increase adhesion of NK cells to ICAM-1 in an LFA-1 dependent manner. (A) Expression of immobilized ICAM-1 on the surface of cell sized beads. Cell sized beads were immobilized with the indicated amounts of mouse ICAM-1 ($\mu\text{g}/10^7$ beads); then the amount of ICAM-1 immobilized was determined using flow cytometry. Shaded and unshaded histograms represent the relative fluorescence of detected ICAM-1 or BSA (0 μg of ICAM-1) using anti-ICAM-1 specific antibodies, respectively. (B) The mean fluorescence intensities (MFI) for ICAM-1 immobilized on cell-sized beads was plotted against the concentrations of ICAM-1. The solid line represents the plot representing the MFI vs ICAM-1 concentration, while the dotted line represents the graph's trendline. (C and D) NK adhesion was measured following the crosslinking of the indicated primary antibody using $\gamma\alpha\text{r}$ or $\gamma\alpha\text{m}$ to BSA beads, (0 μg of ICAM-1), or increasing amounts of immobilized ICAM-1 (at the indicated concentrations) for 3 min. These results are representative of at least two independent experiments. (E) An expanded view for NK cell adhesion to ICAM-1 beads of data from panel C (0-0.5 μg ICAM-1/ 10^7), following stimulation of NK cells with anti-Ly49D, CD16, NKG2D, or MHC I mAbs.

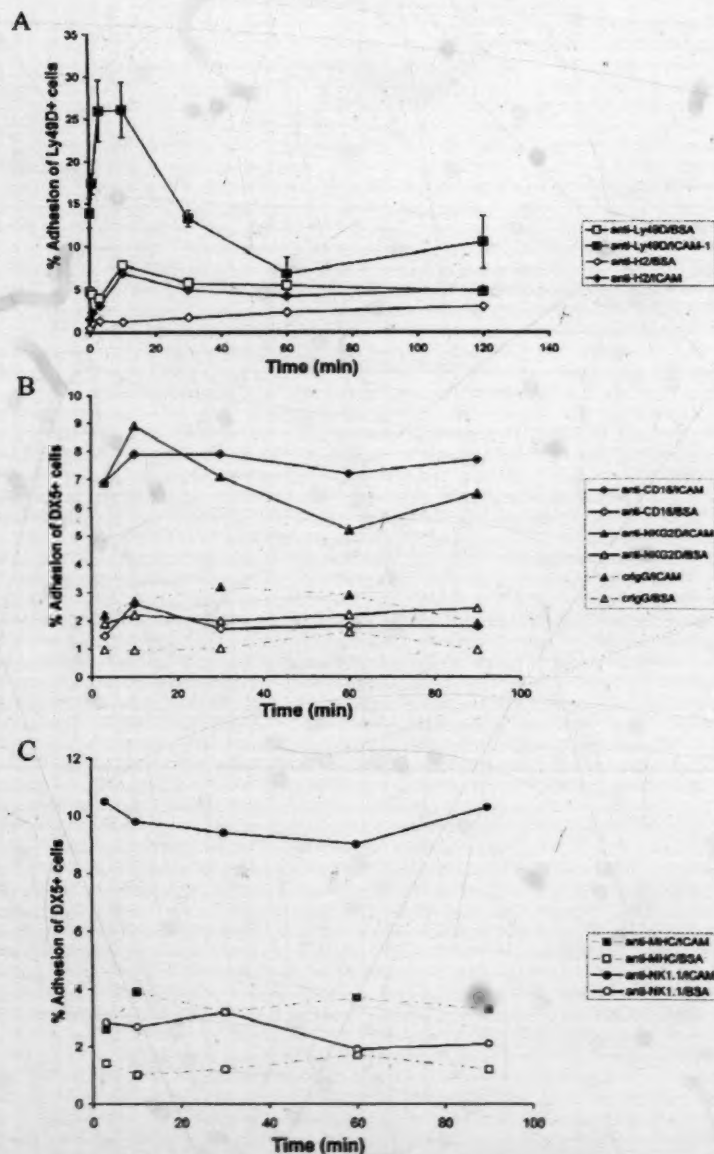


Figure 4-3. Ly-49D induces a very rapid but transient and substantial adhesion of NK cells to ICAM-1 beads, while CD16, NKG2D, and NKR1-1 induce a mild but sustained level of adhesion. NK cell adhesion to BSA or ICAM-1 ($2 \mu\text{g}/10^7$) beads was measured in response to crosslinking of (A) Ly-49D, (B) CD16 or NKG2D, and (C) NKR1-1, or the corresponding rat or mouse isotype matched controls, with secondary antibodies, for the indicated times at 37°C . These results are representative of three (panels A or B) or two (panel C) independent experiments, respectively.

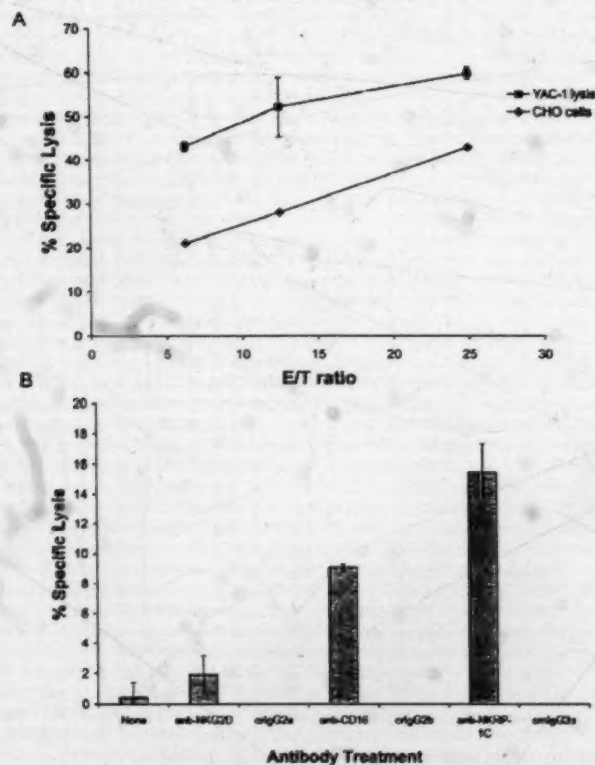


Figure 4-4. Ly-49D, CD16, and NKRP-1C can trigger a cytotoxic NK response in *ex vivo* NK cells. *Ex vivo* DX5-enriched NK cells were used as effector cells in a (A) ^{51}Cr -release assay using YAC-1 or CHO cells as targets. (B) DX5-enriched *ex vivo* NK cells were used in a redirected cytolysis assay with human FcR^+ Daudi target cells (E/T 6/1) pre-loaded with the antibodies recognizing the specific receptor. These results represent the mean values $\pm$ SD.

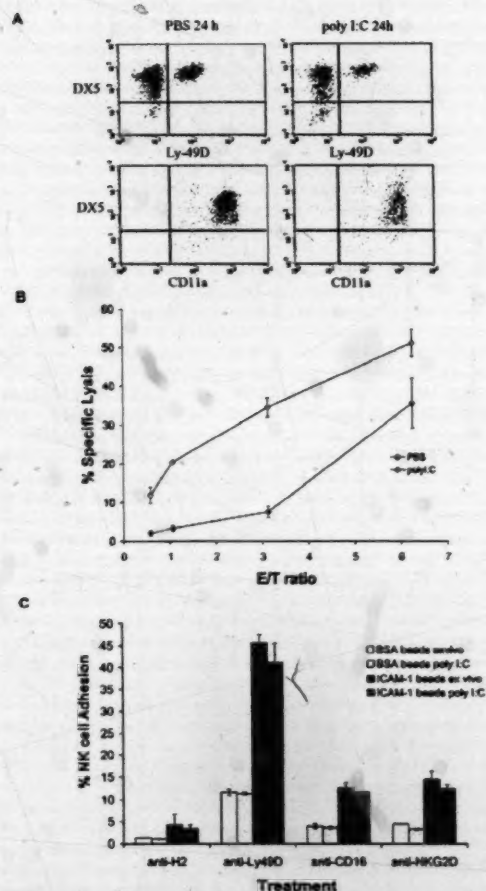


Figure 4-5. NK priming *in vivo* does not increase activating receptor-induced adhesion of LFA-1 for ICAM-1. C57BL/6 mice were injected i.p. with PBS or 50 μ g of poly I:C in PBS for 20 h, then DX5+ cells were purified and (A) used for the detection of surface Ly-49D, or LFA-1; (B) cytotoxicity of YAC-1 tumor target cells; or (C) the Ly-49D, NKG2D, or CD16-induced adhesion to BSA or ICAM-1 ($2\mu\text{g}/10^7$) beads at 3 min at 37 ° C. These results are representative of two independent experiments with error bars representing mean $\pm$ SD.

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Chapter 5 - General Discussion and Conclusions

A. Ly-49 Allele specificity – a role for the $\beta 4$ - $\beta 5$ loop and CK-1

Ly-49 receptors recognize MHC I in an allele specific manner (1-3). As previously discussed, the “site 2” between the CRD of the Ly-49A and its H-2D^d ligand is the region for Ly-49 recognition of MHC I (4, 5). The specificity of Ly-49 receptors for MHC I is dictated by amino acids within the $\beta 4$ - $\beta 5$ loop as highlighted by studies involving the reciprocal substitution of amino acids between two Ly-49 receptors with different MHC I specificities resulted in the transfer of corresponding receptor specificities (6).

The $\beta 4$ - $\beta 5$ loop may contribute to or define the specificities of MHC I allele recognition for Ly-49A and its related receptors (ex. Ly-49P, Ly-49D, Ly-49W, Ly-49G, Ly-49L). These receptors share a group of solvent exposed residues primarily residing within the $\beta 4$ - $\beta 5$ loop that interact with class I and $\beta 2m$ at site 2 (7). In chapter two, I identified the epitope for the CK-1 mAb to a region lying within the $\beta 4$ - $\beta 5$ loop of Ly49GB⁶. In addition, CK-1 was clearly able to disrupt the interaction between Ly-49G^{B6} and its H-2D^d ligand – suggesting that CK-1 was binding at the $\beta 4$ - $\beta 5$ loop and disrupting MHC I recognition. These results further emphasize the importance of the $\beta 4$ - $\beta 5$ loop in regulating Ly-49 MHC I allele specificity.

B. CK-1 and Cwy-3 are useful for studying Ly-49 receptors *in vitro* and *in vivo*

The identification and characterization of the CK-1 and Cwy-3 mAb epitopes will be useful in studying Ly-49 receptors with CK-1 or Cwy-3 epitopes. For example, Ly-49M is an activating receptor expressed in the NOD mouse strain (Table 1-2) that is stained by all available anti-Ly-49G mAbs – CK-1, Cwy-3 and 4D11 (Fig. 2-1). NK cells from NOD mice probably express Ly-49G, Ly-49M, or Ly-49W individually, or in combination, as the cDNAs encoding these receptors were cloned from IL-2 activated NOD NK cells. Ly-49M and Ly-49G but not Ly-49W are recognized by CK-1, and Ly-49M and Ly-49W are recognized by Cwy-3 (Fig. 2-1, Supp. Fig. 2-2). Thus, a combinatorial antibody

strategy can be employed to examine NOD NK cell subsets expressing Ly-49M⁺Ly-49G⁺Ly-49W⁺ cells, as NK cells simultaneously expressing all three receptors will stain positive for both antibodies, in addition to NK cells that are only positive for Ly-49M (as predicted using Fig. 2-1).

Studies using the NOD mouse strain have mapped the type I diabetes susceptibility locus *Idd6* to a region in the NKC (8). NOD NK cell *in vivo* activation through the chronic injection of poly I:C has been associated with reduction in the onset of type I diabetes (9). Thus, the role of NK cell subsets expressing Ly-49G, Ly-49M, or Ly-49W can be closely examined using these antibodies. For example, these antibodies can be used to selectively deplete Ly-49M⁺ and Ly-49W⁺ or Ly-49G⁺ and Ly-49M⁺ cells *in vivo* using Cwy-3 or CK-1, respectively. Alternatively, Ly-49M/Ly-49W or Ly-49G/Ly-49M can be simultaneously co-crosslinked on NK cell or T cell subsets expressing these receptor combinations via Cwy-3 or CK-1, respectively, to measure LFA-1 triggering (10), cytokine production (11) or NK cytotoxic granule release (12).

As the number of identified *Ly-49* receptors are rapidly expanding, CK-1 and Cwy-3 can be used to stain and examine the surface expression of unidentified receptors that share the protein sequences composing their epitopes. Alternatively, the proper protein folding of known *Ly-49* receptors subjected to site directed mutagenesis can be monitored using either Cwy-3 or CK-1. For example, Ly-49W is not recognized by CK-1 but is recognized by Cwy-3 and 4D11 (Fig. 2-1). In order to ensure proper protein folding, both 4D11 and Cwy-3 can be employed to ensure that the proper global protein conformations have been adapted by virtue of the preservation of their respective epitopes. Furthermore, the expression of *Ly-49G*, *Ly-49W*, or *Ly-49M* alleles or the function of NK cell subsets expressing these receptors from F₁ animals (e.g. NOD, and BALB/c) can be examined using both CK-1 and Cwy-3 as previously employed by the AT8 and Cwy-3 antibodies (13).

C. Activating Ly-49 receptors lower the threshold for *ex vivo* NK activation by regulating LFA-1-dependent adhesion

The binding affinities for activating and inhibitory immune receptors sharing the same ligand illustrate an interesting paradigm present in immune cells. In many cases, inhibitory receptors have evolved to possess a greater intrinsic binding capacity for their ligand than activating receptors sharing that ligand; however, in some cases this observation is not evident. The Ig-like CD28 and cytotoxic T lymphocyte associated protein-4 (CTLA-4) receptors are prototypical examples highlighting this phenomenon. Both CD28 and CTLA-4 are expressed on the surfaces of T cells, and recognize the B7 family of receptors present on antigen presenting cells. CD28 provides resting T cells with a positive co-stimulatory signal for cell activation, while CTLA-4 provides a negative signal (14). Interestingly, CTLA-4 is expressed on the surface of only activated T cells, and binds to B7 with a much higher affinity than CD28 ($K_d \sim 12$ nM and 200 nM, respectively) (14), thereby outcompeting CD28 for B7 when both receptors are present.

A similar situation is evident with activating and inhibitory MHC I-specific NK receptors. As previously discussed, certain activating KIR are highly homologous to inhibitory KIR within their extracellular domains, and share the same MHC I ligand. For example, the activating KIR2DS1 and the inhibitory KIR2DL1 both bind to the HLA-Cw4 allele product (15). KIR2DL1 interacts with HLA-Cw4 with a much higher affinity than KIR2DS1 (16). Interestingly, the affinity of KIR2DS1 can be substantially augmented by a single amino acid substitution in the extra-cellular domain (16). Although there are no direct studies assessing the differential binding affinities of activating and inhibitory Ly-49 for the same MHC I allele, several indirect observations have suggested that activating Ly-49, like activating KIR, are not as effective as inhibitory Ly-49 in binding to their ligands. For example, Ly-49A and Ly-49D both recognize H-2D^d (1, 17). When H-2D^d is expressed in plate-adhered COS-7 cells, Ly-49A⁺ cells bind to these cells at much higher levels than Ly-49D⁺ cells (18). In contrast, the binding capacity of Ly-49D for H-2D^d tetramers is substantially enhanced upon

the elimination of an N-linked glycosylation site within the CRD region (19). These studies, however, did not directly measure the binding affinities of Ly-49A and Ly-49D, or Ly-49D glycosylation mutants for H-2D^d. Similarly, surface plasmon resonance studies assessing the binding affinities of activating Ly-49H and inhibitory Ly-49I for the MCMV MHC-like m157 gene product have demonstrated that the Ly-49H affinity for m157 was substantially lower than that determined for inhibitory Ly-49I, suggesting that the MHC-like m157 viral protein has evolved to quell NK cell activation by expressing a ligand that can bind the inhibitory Ly-49I more effectively than activating Ly-49H, resulting in a dominant negative signal (20).

From our studies, we have defined the regulation of NK cell adhesion to susceptible target cells as a novel function of MHC I specific NK activating receptors. We showed that Ly-49P stably expressed in RNK-16 cells substantially increases NK cell adhesion and cytotoxicity to cells expressing its H-2D^d ligand in an Ly-49/MHC I and LFA-1/ICAM-1 dependent manner (Fig. 3-2). Moreover, crosslinking the cell surface activating Ly-49 with a soluble antibody mimics this phenomenon as cells expressing a wild type Ly-49, but not cells expressing a mutant receptor unable to associate with DAP12, have an enhanced binding to plates or cell-sized beads coated with purified ICAM-1 (Fig. 3-4, 3-5). Together, these studies highlight the regulation of LFA-1 binding of ICAM-1 by DAP12 associated Ly-49 receptors through inside-out signaling. This principle can be extended to include human cells because I have also shown that IL-2 activated KIR2DS1⁺ human NK clones increase the adhesion and cytotoxicity of NK cells to target cells expressing the KIR HLA ligand in a KIR-dependent manner (Appendix 3).

The regulation of LFA-1 by inside-out signaling through activating Ly-49/DAP12 receptor complexes may provide a mechanism that compensates for the apparent lower affinity, when compared to their inhibitory receptor counterparts, of activating receptors for their ligands. Upon the activation of Ly-49/DAP12 signals by soluble antibody crosslinking or ligand recognition, inside-out signals acting on LFA-1 triggers its ICAM-1 binding capacity. As there are

no mouse specific serological reagents that can distinguish between the different LFA-1 forms, we have not definitively determined the form adopted by LFA-1 in response to Ly-49/DAP12 stimulation. Our studies strongly suggest that a higher affinity form is adopted as the level of cell/cell, cell/ICAM-1 bead, or cell/ICAM-1 immobilized beads is substantially induced (Fig. 3-2, 3-4, 3-5). LFA-1 adopts three conformations – a low affinity form ($K_d \sim 1\text{mM}$) present on circulating lymphocytes; an intermediate affinity form ($K_d \sim 10\text{ }\mu\text{M}$) that is laterally mobile and is induced by inside-out signals from antigen receptors such as the TcR; and a high affinity form ($K_d \sim 100\text{ nM}$) that forms stable interactions with ICAM-1, and promotes tight adhesion between lymphocytes and cells expressing ICAM-1 (21).

Activating receptors which associate with ITAM-containing signaling adapters have different affinities for their ligands, e.g. TCR $K_a\ 10^3\text{-}10^5\text{ M}^{-1}$ (22), FcγRIII for IC $K_a\ 10^7\text{ M}^{-1}$ (23), BCR $K_a\ 10^6\text{-}10^{11}\text{ M}^{-1}$ (24). Receptors that have lower intrinsic binding affinities for their ligands, such as the TCR or activating Ly-49, may require increased adhesion of LFA-1 for ICAM-1 in order to generate a productive signal. Tight and sustained adhesion between LFA-1 and ICAM-1 would facilitate the multiple engagement of different receptors with a single ligand to trigger a productive signaling cascade as evident with the TCR (25). This suggestion is corroborated by our observations indicating that the Ly-49D/DAP12 receptor complex is capable of triggering tight adhesion to low densities of ICAM-1 on *ex vivo* NK cells (Fig. 4-2C). The triggered activation of LFA-1 adhesion by the Ly-49/DAP12 receptor complex induces a rapid but transient upregulation of LFA-1-dependent tight adhesion peaking at 3 min, that is substantially reduced by 30 min (Fig. 4-3A). The kinetics observed for Ly-49D/DAP12 mediated LFA-1/ICAM-1 tight adhesion are in line with the kinetics observed for cytotoxicity, as several groups have reported that the rate of NK cell mediated cytotoxicity declines after 1 hour, and cytotoxic granule release, through the detection of cell surface CD107a, is also significantly detected within 30 min (12, 26-28), although the kinetics of Ly-49 mediated degranulation have not been established. Similarly, the TCR/CD3 complex has also been shown to induce a

transient upregulation of LFA-1 binding for ICAM-1 that peaks after 30 min (29), and cytotoxic granule release by CTL is also detectable within 30 min (30).

Upon close examination of some of the signaling mediators required for the regulation of Ly-49/DAP12 of LFA-1 triggered adhesion, certain key players play a prominent role. From our studies, it is clear that the association between DAP12 and the activating Ly-49 is required for this event (**Fig. 3-4A**). In addition, SFKs and Syk family kinases are important for tight adhesion (**Fig. 3B**), and Ly-49-mediated cytotoxicity (**Appendix 2**). Furthermore, biochemical studies examining wild type NK cells or NK cells derived from CD45 deficient mice have suggested that the SFK p56^{Lck} and p59^{Fyn} are important effectors in Ly-49 activating receptor membrane proximal signaling events (31). Together, these studies highlight the importance of receptor recognition, and membrane proximal signaling events such as DAP12 association, SFK phosphorylation of DAP12 ITAMs, and Syk family kinase activity in regulating inside-out activation of LFA-1 adhesion and cytolytic responses in NK cells (See **Fig. 5-2**).

Upon antigen receptor engagement, LFA-1 is recruited to the site of cell/cell contact through a variety of mechanisms where it can increase cellular adhesion through its interaction with ICAM-1, or it can provide biochemical signals which can further potentiate signals generated by the antigen receptor complex. For example, LFA-1 can generate co-stimulatory signals for both CD4⁺ and CD8⁺ T cells through PI3K activity leading to enhanced proliferation (32). In addition, studies using peripheral blood T cells have suggested that LFA-1-dependent signaling events directly initiate actin polymerization and reorganization to strengthen its adhesion to ICAM-1 (33). In fact, it has recently been suggested that LFA-1 signals generate an "actin cloud" – or a region above the contact site highly enriched in actin, requiring the activities of the signaling proteins ADAP and c-Jun N-terminal kinase (JNK), which augment T-cell activation and lower the threshold required for T cell activation (34).

Upon its activation, high affinity forms of LFA-1 at the site of cell contact are preferentially enriched in lipid rafts (35), or cholesterol-rich detergent insoluble microdomains. These regions are enriched in a number of signaling

molecules such as SFK (e.g. p56^{Lck}, p59^{Fyn}), and the adapter proteins LAT and NTAL, shown to be important for T and NK cell activation (36, 37). In fact, it has been suggested that chemotactic signals induce the internalization of LFA-1 from the cell surface into recycling endosomes, and subsequent LFA-1 targeting to rafts (38). LFA-1 enriched in rafts, can in turn generate outside-in signals. For example, LFA-1 can generate its own unique signal in NK cells, and perhaps CTL, as it induces the polarization of cytotoxic granules towards targets cells (39).

Several studies have suggested a role for Syk in regulating integrin outside-in signaling. For instance, crosslinking LFA-1 in neutrophils results in neutrophil degranulation, in contrast, cells derived from Syk^{-/-} mice are unable to release oxidative mediators (40). In addition, integrin crosslinking also results in the co-localization of Syk with CD18, and enhanced Syk phosphorylation (40). Similarly, Syk is required for chemokine-dependent B cell polarization (41). Moreover, it has recently been suggested that the ITAM containing adapters DAP12 and FcR- γ are required for integrin outside-in signaling as macrophages and neutrophils deficient in DAP12 and FcR- γ have a reduction in migration, and degranulation in response to integrin crosslinking (42). In other words, important effector molecules required for the inside-out activation of LFA-1 (e.g. Syk, DAP12) are also required for LFA-1 outside-in functions following the binding of LFA-1 for its ligands. Because lipid rafts are enriched in SFKs, activated forms of LFA-1 and Syk, it can be suggested that these microdomains may provide a link that can directly modulate integrin activity. NK activating receptors, for example, may be enriched or actively targeted to rafts following ligand recognition, thereby recruiting signaling effectors to rafts and promoting outside-in signals. This idea is supported by the observation that NKG2D is enriched in rafts following its recognition of target cells expressing MIC-A in human NK cells (43); however, the enrichment of activating Ly-49 receptors in rafts has not been established. In other words, positive signals from activating receptors can prime NK cells for generating LFA-1-dependent outside-in signals in rafts that together may synergize to regulate cytotoxicity, and NK effector functions.

From our results, it can be suggested that the activation of LFA-1 lowers the threshold required for NK cell effector functions, as activating Ly-49 receptors provide signals that generate a form of LFA-1 that binds to ICAM-1 with high affinity – a form presumably enriched in lipid rafts. Our studies support a model where LFA-1 activation and NK cytotoxicity require the presence of an additional activating signal provided by Ly-49/DAP12 receptor complexes (Fig. 5-2). We propose that the combined signals generated from the activated form of LFA-1 and from the activating Ly-49 receptor synergize to overcome the signaling barrier required for cellular activation. This model is supported by our observation that *ex vivo* NK cells do not bind to ICAM-1 at a high frequency in the absence of Ly-49 stimuli, and Ly-49/DAP12-dependent destruction of target cells requires the engagement of LFA-1 with ICAM-1. It is also supported by studies from Eric Long's laboratory suggesting that LFA-1 provides signals in freshly isolated NK cells that promote cytotoxic granule polarization, but not degranulation (39). Similar models have been proposed using other lymphocytes. For example, studies using B cells have suggested a role for the interaction between LFA-1 and ICAM-1 in lowering the threshold required for B cell activation by lowering the level of specific antigen required to form a mature immunological synapse (24). Similarly, LFA-1 engagement of ICAM-1 has been shown to lower the threshold required for T cell activation through a cytohesin/JAB – dependent mechanism (44) – suggesting that the regulation of lymphocyte adhesion receptors by antigen receptors may be a pervasive mechanism in lowering the threshold required for the activation of unstimulated lymphocytes.

D. Non-DAP12 associated NK receptors regulate increased LFA-1 binding to ICAM-1 – a unique role for activating Ly-49 or DAP12?

From our studies examining CD16, NKRP-1C, and NKG2D, it is clear that these receptors are able to modulate LFA-1 binding to ICAM-1 following receptor crosslinking. It is peculiar that only 10-12 % of the cells participate in binding to ICAM-1 despite the relatively high frequency of expression of these receptors on *ex vivo* NK cells (Fig. 4-1, 4-2). In addition, it is highly interesting

how the adhesion they generate is long lived kinetically, and is clearly distinct from the behavior of the Ly-49D/DAP12 receptor complex that induces a rapid but transient increase in the frequency of LFA-1 triggered adhesion to ICAM-1. These observations may stem from a synchronized response by the Ly-49D⁺ NK cell subset when saturating concentrations of anti-Ly49D mAb are used. Whereas, cells expressing CD16, NKG2D, or NKRP-1C may be sequentially triggered to increase their LFA-1 adhesion to ICAM-1 when their receptors are crosslinked with saturating mAb.

From our studies using RNK cells stably expressing a mutant receptor unable to associate with DAP12 (RNK-P(R57L)), it can be suggested that Ly-49 driven inside-out signaling requires the association of DAP12, and is likely mediated through a DAP12-dependent mechanism. We have not examined inside-out signaling using *ex vivo* NK cells derived from DAP12 deficient mice because Ly-49D surface expression is significantly reduced (45). In addition, although *ex vivo* NK cells from mice expressing DAP12 containing an ITAM^{null} version express normal cell surface levels of Ly-49D, studies using cells from these mice will only highlight the importance of Tyr residues within the ITAM for Ly-49 induced inside-out signals (46). Interestingly, DAP12 shares a high degree of amino acid similarity with FcR- γ , but is not similar to DAP10. Both DAP12 and FcR- γ signaling adapters are disulfide linked homodimers expressing a single ITAM within the cytoplasmic tail of each monomer, whereas DAP10 does not contain an ITAM, but uses a YxNM motif to activate PI3K signaling (reviewed in (47)). In addition, DAP10 employs Vav-1 to mediate its cytotoxicity, while both FcR- γ and DAP12 cytotoxic signaling require Vav-2 and Vav-3 as highlighted from studies using mice deficient in these signaling proteins (48). Furthermore, cytotoxic signals through NKRP-1C, CD16 and Ly-49 receptors complexes require a functional SFK (31, 49). Thus, it is highly interesting that the Ly-49D receptor, which associates with DAP12, behaves very differently with respect to regulating adhesion from the CD16 or NKRP-1C receptors, which both associate with the FcR- γ signaling chain.

The differences we observe between Ly-49/DAP12 and NKRP-1C/FcR- γ or CD16/FcR- γ receptor complexes with respect to LFA-1 mediated adhesion may stem from differences inherent to the cells expressing these receptors as a consequence of undefined developmental predispositions, or they may result from an undefined property inherent to DAP12 or the Ly-49 receptors. For example, NK cells that develop in a host deficient in MHC I, or cells that do not express an inhibitory Ly-49 receptor that recognizes self-MHC I exhibit a substantially reduced functionality when compared to NK cells expressing an inhibitory receptor for self MHC I in an MHC I sufficient environment (11, 50). These responding or "licensed" NK cells express the Ly-49C and Ly-49I inhibitory receptors specific for self-MHC I in a B6 mouse (H-2^b) (11). Interestingly, NK cells from B6 mice expressing Ly-49H are also predisposed to express Ly-49D, as approximately 70 % of the Ly-49H⁺ cells are also Ly-49D⁺ (51). In addition, about 68 % of the Ly-49H⁺ cells are also Ly-49C/I⁺ (51). In other words, Ly-49D⁺ cells are also polarized to be Ly-49H⁺, and might have a large fraction of "licensed" (approximately 48 %) cells by virtue of their expression of inhibitory receptors specific for self-MHC I. Thus, it may be possible that licensed NK cells within the Ly-49D⁺ NK cell subset may be pre-disposed to regulate LFA-1 mediated adhesion and also responding upon the cross-linking of other activating receptors, such as through CD16, NKG2D, or NKRP-1C. These ideas can simply be tested by staining CD16, NKRP-1C, or NKG2D cross-linked B6 NK cells co-incubated with ICAM-1 beads with anti-Ly-49D and/or anti-Ly-49H mAbs, and by staining Ly-49D crosslinked B6 NK cells co-incubated with ICAM-1 with anti-Ly-49C/I mAb.

Alternatively, DAP12 may play a unique role in NK cells that has not been defined. DAP12 is a relatively short polypeptide of only 113–114 amino acids, containing a cytoplasmic ITAM. The ITAM is the only signaling domain that has been identified in the DAP12 polypeptide, and it mediates all of its known effector functions to date (46). When the sequence of its cytoplasmic tail is compared with the FcR- γ signaling adapter, it is apparent that the sequence TRKxR/H eight amino acids from the ITAM is conserved in DAP12, but not FcR-

γ . Studies using the NK activating receptor DNAM-1 have shown that the C-terminal end of the cytoplasmic tail of this receptor contains the conserved sequence (between mouse and human) SRRPK, a sequence corresponding to the consensus site for protein kinase C phosphorylation. DNAM-1 constitutively associates with LFA-1 in human NK cells, and can inducibly associate with LFA-1 following in lipid rafts anti-TCR crosslinking in T cells (52). The association in T cells requires the phosphorylation of the Ser residue in the SRRPK sequence (52). In other words, an additional motif unique to the DAP12 cytoplasmic tail may be responsible for regulating LFA-1 inside-out activation, possibly through a protein kinase C dependent mechanism.

A different but interesting idea that may explain the observed difference between Ly-49D and NKRP-1C, CD16, or NKG2D receptors may stem from the enrichment of Ly-49D in lipid rafts. Upon close examination of activating Ly-49 receptor sequences, a membrane proximal cytosolic Val-Cys-Ser sequence permissive for the reversible addition of palmitic acid moieties is present (in Ly49D, H, P, M and W), but not present in CD16, NKRP-1C or NKG2D. Proteins enriched in lipid rafts, such as LAT, are highly palmitoylated, and palmitoylation is important for their raft recruitment (53). In addition, the initiation of TCR membrane proximal signaling events following the recognition of peptide MHC complexes requires the activities of proteins resident to lipid rafts, such as SFK, and LAT (reviewed in (54)). Thus activating Ly-49 constitutive, or induced palmitoylation, may enhance and amplify signals generated from these receptor complexes by recruiting them into microdomains enriched in signaling proteins and activated LFA-1, which can in turn enhance LFA-1 dependent outside-in signals.

E. Future Directions:

Activating NK Receptors and the Regulation of LFA-1 Adhesion

Inside-out activation of LFA-1 results in changes in LFA-1 affinity, or lateral mobility in the membrane. Studies conducted in T cells have identified some of the key players involved in these processes which include the proteins talin, Rap-1, LAT (55, 56), SLP-76 (57) and ADAP(58). TCR signaling uses

LAT to recruit and activate SLP-76 thereby linking to ADAP, with the latter specifically required for LFA-1 clustering (reviewed in (59)). ADAP, in turn activates Rap1 through RIAM (60) which recruits RapL to the LFA-1 α chain to increase its clustering and affinity for ICAM-1 (61), (Fig. 5-2). The role(s) of these molecules in the mechanism employed to trigger LFA-1 activation via activating NK receptors has not been defined. In order to examine the roles of these molecules, the use of animals deficient in these signaling molecules followed by the examination of LFA-1 activation would be very informative. For example, the capacity of LFA-1 activation by antigen receptors is severely compromised in Rap-1 deficient B and T cells (62). In addition, the use of ADAP and SLP-76 deficient mice may also be informative. Alternatively, preventing Rap1 activation by the overexpression of a GFP-tagged RapGAP (e.g. Spa I) in RNK-P cells or *ex vivo* NK cells may also be informative in defining the role Rap GTPases play in NK activating receptor mediated inside-out activation of LFA-1 as previously described (63).

Following antigen receptor stimulation, LFA-1 distribution changes from a uniform level to a polarized redistribution profile when detected with confocal microscopy (61). Consequently, it will be important to define the LFA-1 redistribution on RNK cells stably expressing activating Ly-49 receptors and *ex vivo* NK cells using confocal microscopy. In addition, this increased redistribution and commensurate enhancement of "avidity" of LFA-1 can be examined using soluble ICAM-1-Fc (sICAM-1) complexes, as previously described (64), and as employed to examine Ly-49D induced changes in LFA-1 avidity (Appendix 4). Because this assay cannot discriminate between changes in LFA-1 affinity and avidity, it will be necessary to measure changes in affinity using sICAM-1 as previously described (65).

Signaling Adapters and their roles in regulating LFA-1 through inside-out signaling

In order to directly compare the intrinsic capacities of the different signaling adapters, DAP12, DAP10, and FcR- γ which associate with activating NK receptors through an intermolecular interaction for inducing enhanced LFA-1

binding to ICAM-1, it would be important to uncouple the receptor-mediated recognition event from signaling cascades that modulate inside-out signaling. In addition, the heterogeneous expression of different activating receptors as monitored by specific mAbs provides an obstacle when comparing the relative frequencies of LFA-1 mediated triggered cell binding. To overcome these problems, we propose the use of human CD4-fusion proteins that contain extracellular/transmembrane human CD4 domains and the cytoplasmic tails of the different signaling adapters, or a truncated CD4 receptor containing only the extracellular/transmembrane domains, then stably expressing the individual receptors at similar levels in RNK-16 cells (**Fig. 5-3**). Thus, a single and common mAb stimulus through anti-CD4 can be used to trigger the activation of these receptors, and also monitor inside-out signaling through the measurement of LFA-1 dependent adhesion to ICAM-1 in cells expressing the individual fusion proteins. This receptor system may also be useful for defining the LFA-1 surface distribution in the presence of CD4 mAbs and extended to define the exact regions within the cytoplasmic tails of the signaling adapter sequences through the use of deletion mutants. Furthermore, the functionality of these receptors can be determined using redirected lysis assays with P815 targets, which are normally resistant to RNK-16 mediated lysis (**Fig. 3-1**). CD4-fusion proteins of the type described have been used previously in defining the contribution of Grb2 in DAP10 mediated signaling (66), indicating the practical application of such CD4-fusion constructs in the proposed future studies.

Role of palmitoylation in Ly-49 Activating receptor promotion of LFA-1 inside-out signaling

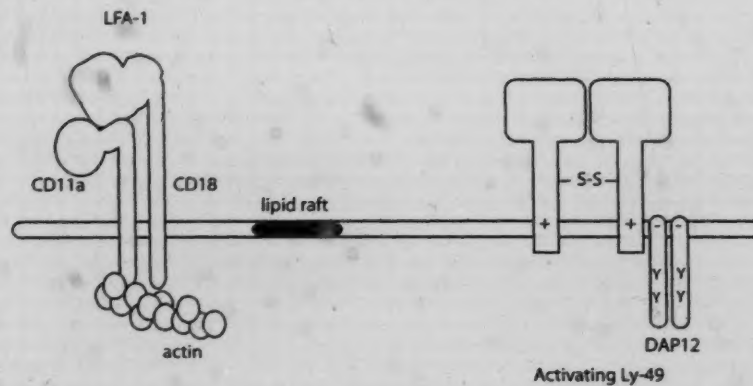
Protein palmitoylation involves the reversible formation of a thioester bond between a free Cys residue, and a palmitate moiety. Thus, in the absence of the accepting free Cys residue, the addition of a palmitate residue is abrogated - resulting in altered protein localization as demonstrated for LAT raft recruitment (67). Interestingly, Ly-49 activators (Ly-49D, H, and W) are expressed, as detected by RT-PCR, as two different splice variants in IL-2 activated NK cells - one form contains a membrane proximal VCS sequence, and the other form does

not (68). To date, only the VCS-containing form of Ly-49P has been reported. Thus, a single point mutation changing the Cys to an Ala mutation within this sequence can be made (**Fig. 5-4**), followed by stably expressing this mutant receptor and the wild type Ly-49P receptor in RNK-16 cells, to assess the ability of cells expressing the individual different receptors to bind to ICAM-1 immobilized plates upon crosslinking Ly-49P. In addition, the role of receptor palmitoylation in inside-out signaling can be corroborated using reversible palmitoyl transferase inhibitors. Furthermore, the palmitoylation of this Cys in the wild type or mutant receptor can be assessed using ^3H -palmitate.

F. Conclusion

By demonstrating that activating NK receptors regulate inside-out activation of LFA-1 binding for ICAM-1, I propose that this is an integral mechanism that lowers the threshold for activating NK cell effector functions. I demonstrated for the first time, that activating Ly-49 receptors, and non-DAP12 associated receptors, regulate LFA-1 with distinct properties. Furthermore, I suggest that activating Ly-49 receptors and DAP12 in NK cells likely parallel the TCR/CD3 complex not only in triggering effector functions, but also likely in the biochemical mechanisms. Moreover, we define the epitopes of the CK-1 and Cwy-3 anti-Ly49G antibodies and underscore their value as useful tools that can be employed for understanding NK cell MHC I allele specificity, and in defining the function of NK cell subsets *in vivo* and the roles they may play in the development of diabetes in the NOD mouse. Finally, I also propose studies that may provide a new dimension in our understanding of NK cell activating receptors and their regulation of LFA-1 triggered adhesion.

A) Circulating NK cell



B) NK cell engaged with target cells expressing Ly-49 ligand

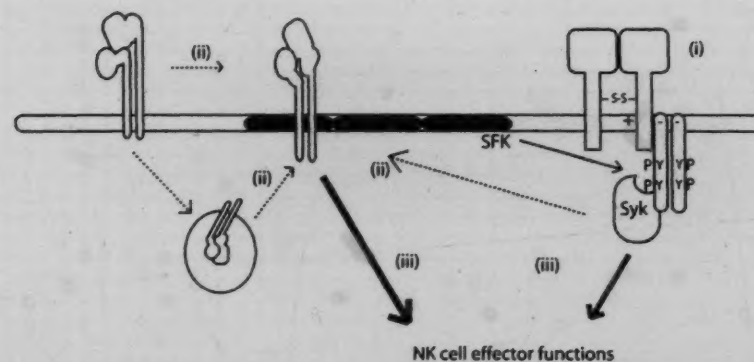


Figure 5-1. Activating Ly-49 receptors lower the threshold for NK cell activation through the induction of inside-out signaling culminating in the activation of LFA-1. (A) Circulating NK cells that have not engaged target cells expressing a ligand for an activating Ly-49 receptor have a form of LFA-1 with a low binding affinity for ICAM-1. (B) (i) Upon ligand recognition, an inside-out signal requiring DAP12 association, and SFK/Syk kinase activity is initiated (ii), which increases LFA-1 lateral mobility, through the liberation from the cytoskeleton and/or the targeting of LFA-1 in recycling endosomes to the site of cell contact, and affinity for ICAM-1. (iii) The activated form of LFA-1 generates signals which combine with the activating Ly-49 derived signals, and together lower the threshold for NK cell activation and trigger NK cell effector functions. The role of inside-out signaling in lowering the threshold for NK cell activation may be extended to include other NK activating receptors such as NKG2D, CD16 and NKRP-1C.

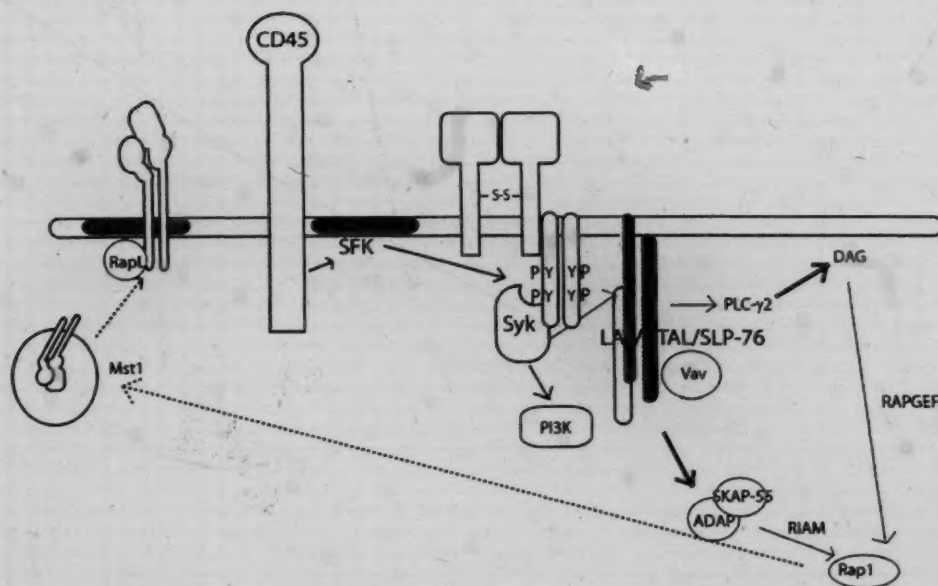


Figure 5-2. Proposed inside-out signaling mediators in response to Ly-49D induction.

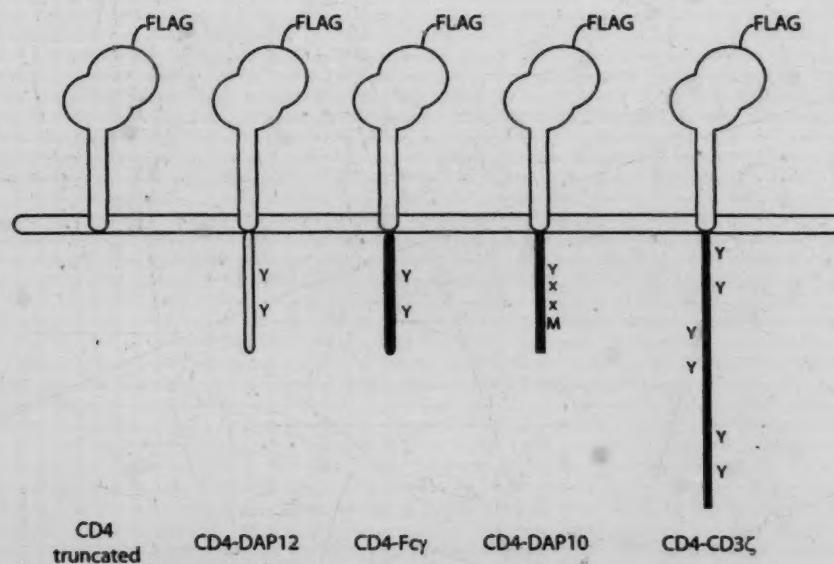


Figure 5-3. Schematic representation of the CD4 chimeric receptors. FLAG-tagged human CD4 extracellular/TM domains were fused with the indicated signaling adapter's cytoplasmic tail.

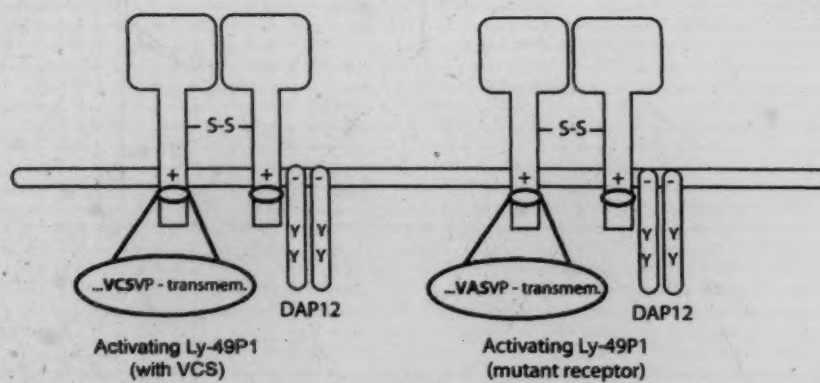


Figure 5-4. Schematic representation of the wild type Ly-49P1 receptor, and a mutant receptor with a C39A amino acid substitution.

G. References

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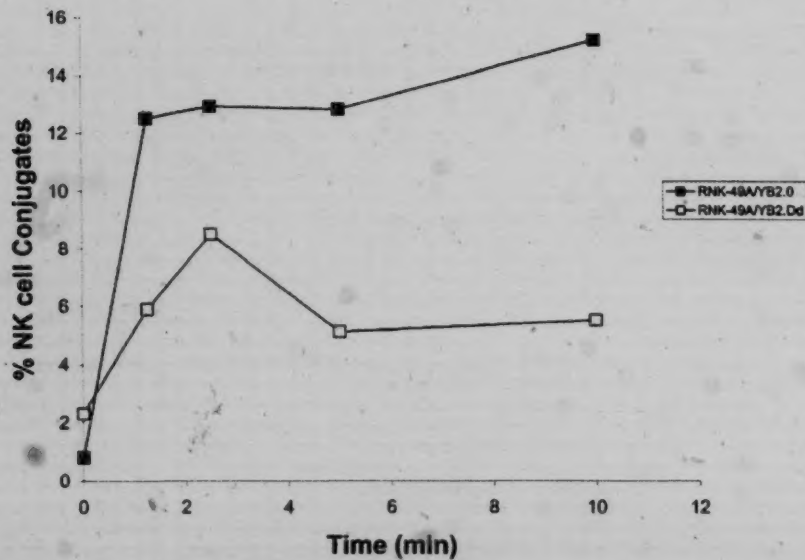
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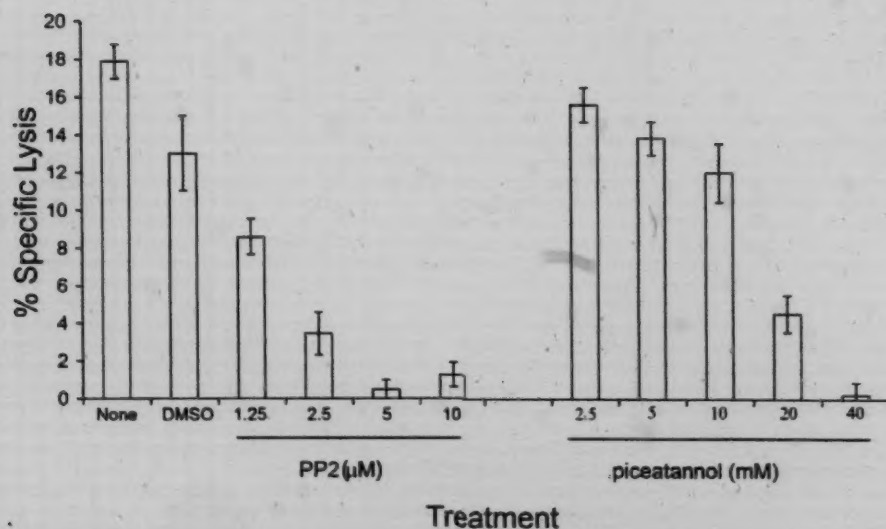
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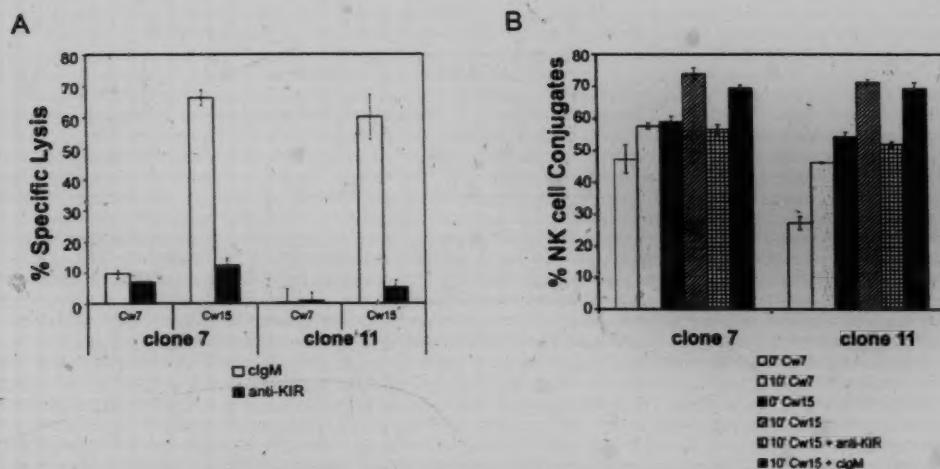
Appendix



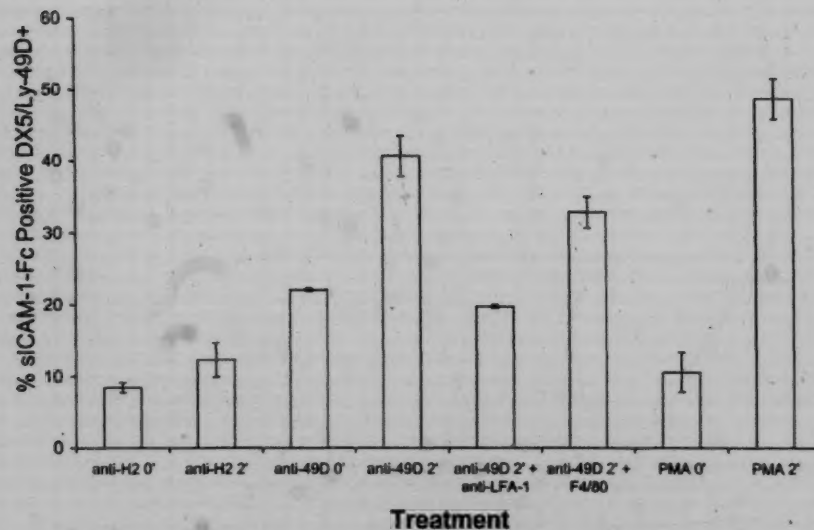
Appendix 1. Ly49A disrupts adhesion between NK cells and susceptible target cells its ligand H-2D^d. RNK-16 cells stably expressing Ly-49A were co-incubated (E/T 2/1) with YB2/0 target cells, or YB2/0 cells stably transfected with H-2D^d, and the percent adhesion was determined over time as outlined in the *Materials and Methods* section in **Chapter 3**.



Appendix 2. Ly-49-mediated lysis requires SFK and Syk kinase activity. RNK-P effector cells were co-incubated with ^{51}Cr -labeled P815 target cells (E/T 12.5/1) in the presence of the indicated concentrations of the SFK inhibitor PP2, or the Syk inhibitor piceatannol as outlined in the *Materials and Methods* section in **Chapter 3**. These results are representative of two independent experiments with results representing the mean values $\pm$ SD.



Appendix 3. Activating KIR increase adhesion of human NK clones to target cells bearing their MHC I ligand. (A) Human KIR2DS positive NK clones specifically lyse 721.221 cells expressing HLA-Cw15, but not HLA-Cw7. Human NK clones were prepared from healthy donors. Selected clones were pre-incubated with HP3E4 (anti-KIR) or control IgM then incubated with ⁵¹Cr labeled Cw7, or Cw15 expressing targets in a ⁵¹Cr release cytotoxicity assay. (B) KIR2D positive human NK clones specifically increase adhesion to susceptible target cells. Selected human NK clones were harvested, labeled with PKH dyes then co-incubated with labeled 221-Cw15 cells (E/T 1/3) for 10 min at 37 ° C. For antibody blocking, cells were incubated with HP3E4 or clgM for 10 mins at RT, targets were added, then incubated at 37 ° C for 10 mins. Data represent the mean of triplicate samples $\pm$ SD.



Appendix 4. Ly-49D crosslinking on *ex vivo* NK cells results in increased LFA-1 affinity and avidity. Changes in LFA-1 avidity were measured as previously described (1). DX5-enriched NK cells were treated with anti-Ly49D or anti-H2 (M1/42) mAb ($1\mu\text{g}/10^6$ cells) and goat secondary mAb ($0.5\mu\text{g}/\text{mL}$), or PMA ($50\mu\text{g}/\text{mL}$) then added to pre-formed sICAM-1-Fc complexes in the presence or absence of either anti-LFA-1 blocking antibodies, or F4/80 ($5\mu\text{g}/\text{mL}$) for 0 or 2 min at 37°C . sICAM-1-Fc complexes were generated by mixing $1\mu\text{g}$ of sICAM-1 with $0.5\mu\text{g}/\text{mL}$ of polyclonal goat anti-human $\text{IgG1-(Fab)}_2^{\text{APC}}$ for each sample in a teflon tube, then diluting to a volume of $10\mu\text{L}$ using PBS containing 1mM MgCl_2 , and 0.9mM CaCl_2 . At the indicated times, NK cells were fixed using room temperature 2% *p*-formaldehyde then the percentage of sICAM-1-Fc complex binding to the cells was assessed. sICAM-1-Fc complex staining of untreated DX5-enriched NK cells were used to determine background levels of sICAM-1 staining, which were subsequently used to normalize the percentage of soluble complex (APC) positive cells within the Ly-49D^+ NK cell subset using three color flow cytometry ($\text{DX5/Ly49D}^{\text{FITC}}/\text{sICAM complex}^{\text{APC}}$). Mean values were derived from duplicate samples, with SD. These results are a representation of two independent experiments.

1. Konstandin, M. H., U. Sester, M. Klemke, T. Weschenfelder, G. H. Wabnitz, and Y. Samstag. 2006. A novel flow-cytometry-based assay for quantification of affinity and avidity changes of integrins. *J Immunol Methods* 310:67.